Vol. 30, No. 2 June, 1963

THE JOURNAL OF DAIRY RESEARCH

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CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price:—TWENTY-FIVE SHILLINGS *Net*
(U.S.A. $4.25)

The Journal of Dairy Research

was established in 1929 as a medium for the publication of the results of original research in dairy science and cognate subjects. It is published by the Cambridge University Press.

The subscription to the *Journal* is 60*s*. per volume, for orders placed in advance of publication, and covers three issues. Single copies may be purchased at 25*s*. each. Postage extra. Subscriptions may be sent to any bookseller or to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. The subscription price in the U.S.A. is $10.00. Enquiries should be sent to the Cambridge University Press, American Branch, 32 East 57th Street, New York 22, U.S.A.

Back numbers of the *Journal* may be obtained from the publishers. Volumes 1 to 4, each part 25*s*. net. Volumes 5 to 28, each single part 25*s*., double parts 50*s*. net. Postage extra. (With the exception of Volume 6, No. 2, Volume 9, No. 1, Volume 10, Volume 11, No. 1 and Volumes 13 and 14 complete, which are out of print.)

THE JOURNAL OF THE

SOCIETY OF DAIRY TECHNOLOGY

17 DEVONSHIRE STREET, LONDON, W.1. *Tel. Langham* 5059

The Journal is issued in quarterly parts, four parts constituting a volume. The parts are usually issued in January, April, July and October each year.

The Subscription rate to non-members of the Society, payable in advance, is £2. 2*s*. 0*d*. net per volume (post free): single numbers, 13*s*. 0*d*. net. Subscriptions and all business enquiries should be sent to the Secretary, Society of Dairy Technology, 17 Devonshire Street. London, W. 1.

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The Journal publishes not only papers read at meetings of the Society, but also papers on dairy technology by members and non-members

Formation of methyl ketones as artifacts during steam distillation of Cheddar cheese and butter-oil

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(*Received* 7 *August* 1962)

Summary. A complete range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} was isolated by steam distillation at atmospheric pressure from fresh butter-oil, cream and Cheddar cheeses of various ages. Evidence was produced to show that the greater part of these methyl ketones was formed during the heat treatment of milk fat. The maximum quantities of methyl ketones obtainable from butter-oil, determined by exhaustive steam distillation at atmospheric pressure, averaged from 14 ppm. for undecan-2-one to 46 ppm. for pentadecan-2-one. Some artifact formation of methyl ketones also occurred, although to much less extent, when dairy products containing milk fat were steam distilled under reduced pressure at 40 °C. Possible mechanisms of formation of the methyl ketones are discussed.

Since it proved possible to extract methyl ketones in low concentration from mature cheese at room temperature, it is evident that milk fat may contain precursors which break down to methyl ketones slowly during cheese ripening, this breakdown being accelerated at higher temperatures. Methyl ketones may therefore play a part in Cheddar flavour, but a previous report from this Institute that the time of first appearance of certain methyl ketones of odd numbers of carbon atoms above C_5 in steam distillates, at atmospheric pressure from Cheddar cheese, coincided with the appearance of the typical Cheddar flavour was not confirmed.

The significance of methyl ketones in relation to the flavour or aroma of cheeses has been investigated by a number of workers. It would appear that these substances are important constituents of the flavour of mould-ripened cheeses (e.g. Morgan & Anderson, 1956) but their contribution to the flavour of other types of cheese is not so clearly established. Harvey & Walker (1960) isolated a range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{11} by steam distillation of Cheddar cheese at atmospheric pressure and considered that their time of first appearance and increase in concentration were correlated with the increase in intensity of the Cheddar flavour as the cheeses matured. Similarly, Jackson (1958), using steam distillation at atmospheric pressure to concentrate the Cheddar flavour, found heptan-2-one, nonan-2-one, butyraldehyde and diacetyl by subsequent gas chromatographic examination. The peaks corresponding to these carbonyls seemed, however, to have no relation to cheese aroma. Other workers (Day, Bassette & Keeney, 1960; Patton, Wong & Forss, 1958; Day & Keeney, 1958) isolated a range of odd-numbered methyl

ketones, as well as other carbonyls, from Cheddar cheese by distillation under reduced pressure at temperatures between 40 and 50 °C.

In contrast, however, to the above findings it is significant that when the extraction of carbonyls was carried out at room temperature (Bassett & Harper, 1958; Kristoffersen & Gould, 1959) no trace of methyl ketones other than acetone was obtained. This suggests that the methyl ketones isolated by other workers may have been artifacts produced by the elevated extraction temperatures. This is a criticism which could apply to all extraction methods involving heating of the cheese slurry, since if methyl ketones are being formed as artifacts at 100 °C, it is also probable that a similar breakdown would occur to some extent at the lower temperatures (40–50 °C) employed by some workers.

The isolation by Patton & Tharp (1959) of the same range of odd-numbered methyl ketones by the distillation of butterfat at 200 °C gives further weight to the possibility of the methyl ketones being artifacts, as does the observation of Bassett & Harper (1958) that the yield of heptan-2-one and nonan-2-one from blue cheese is increased by heating the cheese slurry for 10 min at 100 °C. More recently Boldingh & Taylor (1962) have obtained a range of methyl ketones with odd numbers of carbon atoms from C_7 to C_{15} by the steam distillation at 180 °C of butter-oil, from which volatile carbonyls had been removed completely by high-vacuum degassing.

The possibility of artifact formation was considered by Harvey & Walker (1960), but they concluded that the methyl ketones obtained in their work were not formed during the distillation but were present as such in Cheddar cheese. However, the point did not seem to be clearly established and this investigation was therefore undertaken in an attempt to estimate more precisely the concentration of the various carbonyl compounds in Cheddar cheese and to determine whether, and if so to what extent, methyl ketones were formed as artifacts during distillation.

METHODS

Materials

The cheeses used were manufactured in the Institute factory by normal New Zealand commercial methods. They were kept at 13 °C for 2 weeks and then at 7 °C for the remainder of the ripening period.

Cheese-oil was obtained by grating the cheese, placing it in centrifuge tubes and melting it in a 60 °C water bath. The melted cheese was centrifuged for 5 min at 2000 rev/min and the cheese-oil then poured off. Butter-oil was prepared by melting fresh butterfat in centrifuge tubes and centrifuging as for cheese-oil.

Collection of volatiles

Steam distillation at atmospheric pressure

For experiments with cheeses 1 kg portions (except in preliminary experiments when 500 g were used) were coarsely grated, covered with a litre of distilled water and steam distilled at atmospheric pressure. Preliminary experiments showed that the yield of carbonyls from a cheese was dependent upon the rate of distillation, and in an attempt to standardize the procedure it was therefore arranged that the rate of

distillation was uniform. The distillate was led directly into 100 ml of 2,4-DNP hydrazine (throughout this paper 2,4-DNP denotes 2,4-dinitrophenyl) in 2 N-HCl and the distillate-reagent mixture was then held at room temperature for 24 h. The 2,4-DNP hydrazones were extracted with carbonyl-free chloroform, prepared according to Schwartz & Parks (1961), and the extract evaporated to dryness. The hydrazones were taken up again in the minimum quantity of petroleum ether or hexane for subsequent column chromatography.

For experiments with cheese-oil, 400 g portions were obtained from cheeses as described above. In similar experiments 400 g portions of butter-oil and 1 kg portions of cream were used. The quantities of fat were thus approximately the same in all the materials used.

Steam distillation under reduced pressure

This was carried out as at atmospheric pressure except that the pressure in the apparatus was reduced to keep the temperature of distillation at approximately 40 °C.

Continuous stripping of volatiles from cheese extract with nitrogen at room temperature

1 kg of the cheese was blended with 1500 ml of carbonyl-free benzene. The benzene filtrate was placed in a flask in a closed circuit including a trapping solution of 2,4-DNP hydrazine and a sealed pump (Reciprotor). The apparatus was flushed with nitrogen to displace air and the system was sealed. The nitrogen was circulated continuously for 2 weeks by means of the pump.

Chromatographic separation of 2,4-DNP hydrazones

Initial separation of the 2,4-DNP hydrazones was done in preliminary experiments by the method of Monty (1958) but the column partition chromatography method of Day *et al.* (1960) was subsequently found to be more suitable and was employed for all later work.

Identification of 2,4-DNP hydrazones

Tentative identification of the 2,4-DNP hydrazones was accomplished by comparing retention volumes of the unknown derivatives with those reported by Monty (1958) and Day *et al.* (1960) for known derivatives. The fractions from the column were collected separately and evaporated to dryness for subsequent paper chromatographic analysis. The method of Huelin (1952) was used to identify carbonyls up to C_7 and that of Klein & de Jong (1956) for carbonyls from C_5 to C_{15}. The method of Lynn, Steele & Staple (1956) was used to confirm the identification of the intermediate carbonyls from C_4 to C_9.

Further identification of the 2,4-DNP hydrazones was possible by measurement of their light-absorption maxima in ethanol and particularly by fading studies of their spectra in alkaline ethanolic solution according to Jones, Holmes & Seligman (1956). Particular attention was paid to the subsidiary peak in the 520 mμ region which disappeared with saturated aliphatic aldehydes but persisted with saturated aliphatic ketones. For this purpose a suitable portion of the 2,4-DNP hydrazone of an unknown carbonyl from the column, or spot eluted from a paper chromatogram, was

mixed with 0·25 N-ethanolic sodium hydroxide and the absorption spectrum in the 500–540 mμ region measured immediately and again after an interval of 90 min.

By these methods it was possible not only to identify the methyl ketones present but also to obtain quantitative comparisons of their concentration. In preliminary experiments, methyl ketones not normally found in cheese distillates were added in known concentrations to cheeses of various ages and to butter-oil, and recovery experiments carried out as reported later. The 2,4-DNP hydrazones of the methyl ketones found in the steam distillates could thus be compared directly against markers of known concentration. Because of the large number of samples involved, this method was employed to obtain the exploratory results presented in Table 1, which are therefore semi-quantitative only.

For more accurate quantitative estimations, known weights of the 2,4-DNP hydrazones of each of the methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} were dissolved in alcohol and standard curves prepared relating absorption at 362 mμ to original concentration of methyl ketones. Having identified a methyl ketone it was thus possible to obtain a measure of the quantity present in the distillate. However, as pointed out by Wong, Patton & Forss (1958), a difficulty in the estimation of methyl ketones in this type of investigation is the non-quantitative nature of the reaction of carbonyls with the 2,4-DNP hydrazine reagent. It was not found possible to standardize completely the procedure of steam distillation at atmospheric pressure and reproducibility for the same sample of cheese or of butter-oil was found to be about ±5% for C_7 and C_9 and ±15% for the other methyl ketones. Nevertheless 2,4-DNP hydrazine at present continues to be the most satisfactory reagent available for the isolation and identification of carbonyl compounds.

RESULTS

Steam distillation at atmospheric pressure

In preliminary experiments, steam distillations of cheese slurries were carried out as described by Harvey & Walker (1960), 500 g of cheese being distilled and only the first 200 ml of distillate collected. A range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} was obtained from cheese of all ages with no indication of significant differences in methyl ketone pattern with increasing maturity of the cheeses. It was, however, noted that considerably larger quantities of methyl ketones could be obtained by continuing the distillation for longer periods and that the concentration of methyl ketones in successive 200 ml fractions of distillate did not decrease appreciably until at least 1000 ml of distillate had been collected. Typical results are shown in Table 1.

In contrast to this persistence of methyl ketones with odd numbers of carbon atoms, when known amounts of hexan-2-one, octan-2-one and decan-2-one (even carbon number methyl ketones not normally present in cheeses) were added to water, butter-oil and cheese slurries, decreasing concentrations of these substances were recovered in successive 200 ml fractions of distillate as expected. Traces of these ketones were present in the fourth 200 ml fraction collected but they were not detected in subsequent fractions of distillate. Recoveries were almost quantitative from water and butter-oil and 85–95% from cheese slurries.

These findings suggested that part at least of the methyl ketones, obtainable from cheese by steam distillation at atmospheric pressure, were not present as such in the cheese but were being formed as artifacts during the distillation. Any methyl ketones present in the free state in cheese at least up to C_{11} would be expected to distil over in the first 1000 ml of distillate collected and experiments were carried out in which cheeses of various ages from 1 day to 13 months were distilled, 1 l of distillate being collected and analysed for methyl ketones. As the results shown in

Table 1. *Relative concentrations of carbonyls in successive* 200 *ml fractions of steam distillates at atmospheric pressure from cheese, butter-oil and cream (fat content of each sample approximately* 200 *g)*

			Methyl ketones (mg) in each fraction								Aldehydes	
	Age	200 ml fractions*	C_3	C_4	C_5	C_7	C_9	C_{11}	C_{13}	C_{15}	C_1	C_2
Cheese A	1 day	1st	0·3	0	0·2	0·3	0·1	Tr	Tr	Tr	Tr	0·2
		2nd–5th	0·2	0	0·1	0·4	0·1	Tr	Tr	Tr	0	0
Cheese B	12 days	1st	0·3	Tr	0·2	0·4	0·1	Tr	Tr	0	0	0·1
		2nd–5th	0·1	0	0·1	0·3	0·2	0·1	Tr	Tr	0	0
Cheese C	6 months	1st	0·1	0·2	0·2	0·2	0·1	0·1	0	0	Tr	0·1
		2nd–5th	0·2	0	0·1	0·3	0·2	0·1	Tr	Tr	0	0
Cheese D	13 months	1st	0·2	0·2	0·3	0·4	0·2	0·1	Tr	Tr	Tr	Tr
		2nd–5th	0·1	0	0·2	0·3	0·1	0·1	Tr	Tr	0	0
Cream (40 % butterfat)	From fresh milk	1st–2nd	0·1	0	0·2	0·3	0·2	0·1	Tr	Tr	0	0·2
		3rd–5th	0·2	0	0·2	0·4	0·3	0·1	Tr	Tr	0	0·2
Butter-oil	From freshly made butter	1st–3rd	0·3	0	0·2	0·4	0·2	0·1	Tr	Tr	0	0
		4th–5th	0·3	0	0·1	0·5	0·2	0·1	0·1	Tr	0	0

The grouping of several fractions indicates that each had approximately the same concentration of carbonyls.
Tr = trace, i.e. less than 0·05 mg; 0 = not detected.

Table 2. *Variation in concentration of methyl ketones found in* 1000 *ml steam distillates at atmospheric pressure from cheese, cheese-oil, butter-oil and cream (fat content of each sample approximately* 400 *g)*

		Methyl ketones, mg							
	Age	C_3	C_4	C_5	C_7	C_9	C_{11}	C_{13}	C_{15}
Cheese E	1 day	1·9	0	1·7	4·0	1·1	0·3	0·3	0·1
Cheese F	1 month	1·4	0·1	1·1	3·7	1·2	0·5	0·2	0·2
Oil from Cheese F	1 month	1·4	0	0·3	3·7	1·3	0·5	0·2	0·1
Cheese G	9 months	1·9	0·6	1·1	4·1	1·3	0·6	0·3	0·2
Cheese H	9 months	1·5	0·9	1·8	3·4	1·2	0·5	0·3	0·2
Oil from Cheese H	9 months	1·0	0·3	0·7	3·0	0·8	0·3	0·3	0·3
Cheese D	13 months	1·4	0·3	1·1	3·3	1·5	0·3	0·1	0·1
Butter-oil	1 day	1·9	0·1	1·2	5·4	1·7	0·5	0·3	0·1
Cream	Fresh	1·4	0·1	0·6	3·4	1·3	0·5	0·2	0·2

0 = not detected.

Table 2 indicate, alkan-2-ones with odd numbers of carbon atoms from C_3 to C_{15} were identified in approximately the same total and relative concentrations in each of the cheeses irrespective of age. In general the relative concentrations were heptan-2-one > acetone > pentan-2-one > nonan-2-one > undecan-2-one > tridecan-2-one > pentadecan-2-one. In addition to the five cheeses reported in Table 2, nine other cheeses aged between 1 day and 9 months were examined and showed little variation

from this general pattern. There were, however, significantly higher concentrations of butan-2-one found in mature cheese than in young cheese. That the methyl ketones, or their precursors, were associated with the fat fraction of the cheese was shown by 'oiling off' samples of cheese, and steam distilling the cheese-oil obtained. These cheese-oils yielded the same range and relative concentrations of methyl ketones as did the cheeses from which they had been prepared (Table 2). The association of methyl ketones or their precursors with the fat phase was further demonstrated by the steam distillation at atmospheric pressure of fresh cream (40 % fat) and also of fresh butter-oil obtained by oiling off the butter from similar cream. As Table 2 shows, the same range and relative concentrations of methyl ketones from C_3 to C_{15}

Table 3. *Variation in concentration of methyl ketones found in successive* 1000 *ml fractions of steam distillate at atmospheric pressure of* 1 *kg of a 7-month-old Cheddar cheese*

	Methyl ketones, mg						
Fraction	C_3	C_5	C_7	C_9	C_{11}	C_{13}	C_{15}
1	2·3	2·1	4·8	2·0	1·4	0·3	0·2
2	2·5	1·0	2·1	1·2	1·4	0·4	0·3
3	2·0	0·5	0·6	0·7	1·3	0·6	0·3
4	2·1	0·4	0·4	0·4	0·6	0·6	0·5
5	1·3	Tr	Tr	0·1	0·4	0·6	0·6
6	1·1	Tr	0	Tr	0·2	0·5	0·5
7	1·0	0	0	0	Tr	0·3	0·5
8	0·6	0	0	0	Tr	0·3	0·4
9	0·9	0	0	0	Tr	0·3	0·4
10	0·5	0	0	0	Tr	0·3	0·5

Tr = Trace, i.e. less than 0·1 mg; 0 = not detected.

Table 4. *Variation in concentration of methyl ketones found in successive* 1000 *ml fractions of steam distillate at atmospheric pressure of* 400 *g fresh butter-oil*

	Methyl ketones, mg						
Fraction	C_3	C_5	C_7	C_9	C_{11}	C_{13}	C_{15}
1	2·8	1·9	5·5	1·8	1·1	0·3	0·1
2	2·1	1·0	2·4	1·8	1·9	0·6	0·2
3	2·0	1·2	1·1	1·2	0·9	0·6	0·6
4	1·2	0·5	0·5	1·0	1·1	0·8	0·5
5	1·4	0·4	0·5	0·8	0·9	0·8	0·5
6	1·0	0·5	0·3	0·8	0·8	0·7	0·8
7	0·6	0·3	Tr	0·5	0·6	0·9	0·8
8	0·6	Tr	Tr	Tr	0·2	0·4	0·9
9	0·7	Tr	0	0	Tr	0·3	0·8
10	0·5	Tr	0	0	Tr	0·4	0·6

Tr = trace, i.e. less than 0·1 mg; 0 = not detected.

were obtained as before. Furthermore, successive 200 ml fractions of distillate from cream and butter-oil showed the same pattern of methyl ketone persistence as did comparable fractions from cheeses (Table 1) indicating that the same type of precursor breakdown was probably occurring in cream and butter-oil as in the cheeses.

As shown in Tables 3 and 4, exhaustive steam distillation of cheese or butter-oil results in a slow decrease in concentration of the C_3 to C_{11} methyl ketones in successive

litre fractions of distillate. Individual methyl ketones, however, varied markedly in their persistence, and the close similarity between the two tables affords further evidence of the identity of the precursors of the methyl ketones in butter-oil and in cheese. Thus the 10 l of distillate collected contained all the heptan-2-one and nonan-2-one, present as such or formed as artifacts during heat treatment, and most of the pentan-2-one and undecan-2-one, but recovery of acetone, tridecan-2-one and pentadecan-2-one was still incomplete. Further experiments were therefore carried out in which smaller quantities (10 g) of butter-oil were exhaustively distilled until no further methyl ketones could be detected in 2 l fractions of distillate. In using these smaller quantities of butter-oil the dimensions of the apparatus were correspondingly reduced to ensure effective steam distillation. It was found necessary to collect 10 l of distillate. Total concentrations of methyl ketones so obtained from two separate samples of butter-oil are shown in Table 5.

The results of the above experiments indicated that extraction procedures involving high temperatures were of doubtful value for the estimation of preformed methyl ketones in dairy products containing milk-fat. Alternative methods of extraction involving less drastic heat treatment were therefore investigated.

Table 5. *Concentration of methyl ketones (ppm.) obtained from two butter-oil samples by exhaustive steam distillation at atmospheric pressure*

	100 °C			
	1	2	Average	180 °C*
C_3	45	39	42	—
C_5	16	10	13	—
C_7	28	22	25	12
C_9	20	12	16	9
C_{11}	15	13	14	13
C_{13}	28	20	24	22
C_{15}	51	41	46	42

* As reported by Boldingh & Taylor (1962).

Steam distillation under reduced pressure

Experiments were carried out in which cheeses, cream and butter-oil were steam distilled at 40 °C under reduced pressure. Under these conditions the same range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} was obtained but the yields were very considerably lower than from corresponding samples steam distilled at atmospheric pressure. At 40 °C, however, there was still evidence of the formation of methyl ketones as artifacts. Thus in recovery trials in which 2 mg of octan-2-one was added to butter-oil and cheese, it was found that all the recoverable octan-2-one came over in the first three 250 ml fractions of distillate. In contrast acetone, pentan-2-one and heptan-2-one were present in the fourth 250 ml fraction in approximately the same concentrations as in each of the first three 250 ml fractions indicating, as in the case of steam distillation at 100 °C, that they were being formed as artifacts during distillation. However, the concentrations of methyl ketones found in low-temperature distillates from mature cheeses were greater than those obtained under the same conditions from young cheeses, cream or butter-oil, particularly the

methyl ketones from C_7 to C_{15}. Since the quantities of fat were approximately the same in all samples it is probable that not all of the methyl ketones obtained from mature cheese were formed as artifacts during distillation and that some at least were present as such in the cheeses.

That methyl ketones could be produced by heat treatment of cheese was further demonstrated in an experiment in which 1 kg of a 13-month-old cheese was refluxed for 2 h at 100 °C and then steam distilled at 40 °C under reduced pressure. As shown in Table 6 the preliminary heat treatment increased the yield of methyl ketones very considerably and also altered their relative concentrations. After refluxing, acetone, nonan-2-one and undecan-2-one were the most abundant methyl ketones whereas, in a sample of the same cheese distilled at 40 °C without prior heat treatment, acetone and pentadecan-2-one were found in greatest concentrations.

Table 6. *Effect of prior refluxing on concentrations of methyl ketones found in* 1000 *ml steam distillates obtained under reduced pressure at* 40 °C *from* 1 *kg portions of a* 13*-month-old cheese*

	Methyl ketones, mg						
Portion	C_3	C_5	C_7	C_9	C_{11}	C_{13}	C_{15}
1	0·1	Tr	Tr	Tr	Tr	Tr	0·1
2*	0·9	0·2	0·2	0·4	0·3	0·1	0·1

Tr = trace, i.e. less than 0·1 mg.
* Second portion was refluxed for 2 h at 100 °C before steam distillation.

Continuous stripping of volatiles from cheese extracts with nitrogen at room temperature

Further evidence indicating that free methyl ketones do exist in mature cheese was provided by passing nitrogen continuously through benzene extracts of the cheese for 2 weeks at room temperature. The homologous series of methyl ketones with odd numbers of carbon atoms from C_3 to C_9 was found in a 6-month-old cheese and from C_3 to C_{11} in a 9-month-old cheese. Butan-2-one also was found in both cheeses and hexan-2-one in the 9-month-old cheese. The amounts of methyl ketones obtained were very small, and were of the order of 5% of those obtained by steam distillation at atmospheric pressure. When, however, a further 1 kg of the same 9-month-old cheese containing 2 mg of added heptan-2-one was extracted with benzene, approximately 40% of the heptan-2-one was recovered by passage of nitrogen continuously through the apparatus for 2 weeks. This method of extraction was thus shown to be capable of stripping free methyl ketones from the benzene extract of cheese when they were present. This is further evidence that a considerable portion of the methyl ketones found in distillates at atmospheric pressure are artifacts.

DISCUSSION

The results obtained in the present investigation show that methyl ketones are readily produced as artifacts during extraction procedures which involve the heating of dairy products containing milk fat. Thus on steam distillation at atmospheric pressure, cream, butter-oil, cheese-oil and Cheddar cheeses from 1 day to 13 months old all gave the same range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15}. The persistence during distillation of these substances indicated that

the greater proportions were being formed as artifacts. As shown in Table 5 the concentrations of methyl ketones obtained by exhaustive steam distillation of butter-oil at 100 °C are in agreement with those given by Boldingh & Taylor (1962) for distillation of butter-oil at 180 °C. These appear to be the maximum concentrations of the methyl ketones obtainable by distillation and indicate that the precursors are present in relatively small amounts in milk fat. The concentrations of these precursors would not necessarily be identical in different samples of butter-oil, since the fatty acid composition of different milk fats is not uniform. The average values given in Table 5 are also confirmed by totalling concentrations of methyl ketones found in the successive fractions of cheese and butter-oil (Tables 3 and 4). The close similarity of the values for cheese and butter-oil affords further evidence of the identity of the precursors of the methyl ketones in both products.

Patton & Keeney (1958) have identified pentadecan-2-one and possibly tridecan-2-one in the high-melting glyceride fraction of milk fat, associated with the protein-phospholipid complex in the so-called membrane of milk fat globules. However, in general the rate at which the methyl ketones distil (Tables 3 and 4) would suggest that they are not present in the free state, since, under such circumstances, as distillation proceeds one would expect the exponential decrease in the concentrations of the methyl ketones that normally obtains for simple distillation procedures (Scott, 1955). This is approximately true of heptan-2-one only but not at all for the other methyl ketones.

On the basis of pyrazalone formation with Girard-T reagent, Boldingh & Taylor (1962) suggested that β-keto acids are present in small amounts in bound form in butter. Such keto acids would seem likely precursors of methyl ketones since by simple decarboxylation they would yield methyl ketones containing one less carbon atom. The presence of keto acids with even numbers of carbon atoms from C_4 to C_{16} is consistent with the results of Popják, French, Hunter & Martin (1951) who, analysing milk fat after the intravenous injection of radioactive acetate with a lactating goat, found that fatty acids higher than palmitic possessed very little radioactivity. This was further confirmed by experiments with lactating-rat mammary gland (Dils & Popják, 1962). When optimum conditions for fatty acid synthesis were used all the 'even-numbered' straight chain fatty acids from C_8 to C_{18} were synthesized as well as a small amount of oleic acid. The major components, however, were in the chain length range C_{10}–C_{16}. The generally accepted view (e.g. Folley & McNaught, 1961) is that all fatty acids in milk fat up to and including palmitic are formed mainly by stepwise elongation of a shorter acid by addition of a C_2 compound derived from acetate, whereas C_{18} acids derive mainly from the blood or from precursors other than acetate. It is possible that traces of the keto acids formed from acetate as intermediates in fatty acid synthesis become incorporated into the triglyceride molecule of milk fat, and that they break down on heating to give a range of methyl ketones with odd numbers of carbon atoms up to C_{15}. It seems significant that no heptadecan-2-one could be detected in any of the samples examined despite the relatively large amounts of C_{18} acids in milk fat. This hypothesis is supported by work now in progress in this laboratory with radioactive milk fat (to be reported in detail later). The methyl ketones obtained by steam distillation of milk fat from a cow injected intravenously with radioactive acetate have, with the exception of acetone, been found to be radioactive, indicating that their precursors had been derived from acetate.

Many workers have isolated a homologous series of methyl ketones with odd carbon numbers from cheese distillates. It has been suggested (Harvey & Walker, 1960) that the time of first appearance of certain methyl ketones above C_5 coincided with the appearance of the typical Cheddar flavour. Since such ketones were found not only in distillates from cheese of any age but also from fresh butter-oil it is evident that this is not the case. The above findings throw doubt on some of the evidence which has been advanced in support of the hypothesis that a homologous series of methyl ketones is formed as biological products during Cheddar cheese-ripening. Since, however, methyl ketones are obtained from mature cheese at room temperature, it is possible that the breakdown of precursors which occurs rapidly on heating of milk fat may also take place slowly at ripening temperatures, resulting in the formation of relatively small quantities of a range of methyl ketones as the cheese matures. Some methyl ketones may also be formed by enzyme action during cheese-ripening. For instance butan-2-one was found in comparatively large concentrations in some mature cheeses but not detected in appreciable amounts in butter-oil or young cheese. Scarpellino (1961) has also identified butan-2-one as a major volatile constituent of Cheddar cheese and has reported that an aqueous solution of butan-2-one, butyric acid, acetic acid and methionine gives an aroma akin to that of Cheddar cheese.

It is evident, however, that new methods will have to be employed in the quantitative examination of cheese in order to differentiate between methyl ketones present as such in ripening cheese and those formed as artifacts during the extraction. The further question of whether they play any part in cheese flavour then has still to be answered.

The author gratefully acknowledges the helpful advice of Dr W. A. McGillivray and the technical assistance of Miss Hannah Watt.

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Printed in Great Britain

The determination of citric acid in milk and milk sera

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(*Received* 10 *September* 1962)

Summary. The accuracy of methods that utilize the colour-forming reaction between pyridine, acetic anhydride and citric acid for the determination of citric acid in milk and milk sera has been examined. A method based on that of Saffran & Denstedt (1948), in which a trichloroacetic acid (TCA) filtrate is used, gave values 3–5% low for the citric acid content of both milk and milk ultrafiltrate. The method of Marier & Boulet (1958), in which aqueous samples are analysed, gave an accurate value for the citric acid content of milk ultrafiltrate but a value about 34% high for the citric acid content of milk. The same technique but applied to aqueous–NaOH samples (Marier & Boulet, 1960) gave values about 7% high for the citric acid content of milk and about 3% high for the citric acid content of ultrafiltrate. However, a method using the technique of Marier & Boulet and TCA filtrate gave accurate values for the citric acid content of both milk and milk ultrafiltrate.

As methods for the determination of citric acid in milk that are based on the formation of pentabromoacetone are slow and laborious, methods utilizing the colour-forming reaction between pyridine, acetic anhydride and citric acid have been favoured in recent years. Fürth & Herrmann (1935) appear to have discovered this reaction which, under their experimental conditions, is probably a condensation between pyridine and citraconic anhydride (formed from citric acid via aconitic acid and citraconic acid). Rapid methods based on this reaction were subsequently developed for the determination of citric acid in various biological materials (Saffran & Denstedt, 1948), in cheese starter serum (Evenhuis, 1951) and in milk and milk products (Babad & Shtrikman, 1951). Murthy & Whitney (1957) suggested that the accuracy of the method of Babad & Shtrikman (1951) would be improved if the concentration of trichloroacetic acid (TCA) in the milk-TCA filtrate used for the analysis were increased from 5 to 12% (w/v), and if glass-stoppered instead of rubber-stoppered tubes were used during colour development. In addition, the precision of the method of Babad & Shtrikman was considered unsatisfactory by Reinart & Nesbitt (1957) and they adopted a procedure using a different milk filtrate and the technique of Saffran & Denstedt, slightly modified, for colour development.

Marier & Boulet (1958) found that none of the above methods was satisfactory, and after a thorough investigation of the factors influencing the pyridine-acetic anhydride-citric acid reaction they put forward a convenient procedure that they considered to be of enhanced accuracy, precision and sensitivity. In this method, a mixture consisting of 1 ml of diluted milk (fat-free), 1·3 ml of pyridine and 5·7 ml of acetic

anhydride is held in a water bath at 32 °C for 30 min and the intensity of the yellow colour then measured. When 1 ml of milk-TCA filtrate (12 %, w/v, TCA) was used instead of diluted milk a value about 7 % lower was obtained for the citric acid content of milk; this difference was attributed by these authors to adsorption of citric acid on the protein precipitated by the TCA. Davies (1959) and Evenhuis (1959), however, contended that the difference could result from the use of diluted milk giving high values because of opalescence from undissolved protein in the solution used for colour measurement. In a subsequent publication Marier & Boulet (1960) agreed that the use of diluted milk could result in a slight opalescence but they maintained that the opalescence did not cause a significant positive error. Nevertheless, they altered their procedure by replacing the 1 ml of diluted milk with 0·5 ml of diluted milk plus 0·5 ml of 0·4 N-NaOH to obviate any opalescence, but they held to their view that the lower values obtained by using filtrates from acidified milk resulted from adsorption of citric acid on the precipitated protein. Marier, Boulet & Rose (1961) presented further evidence supporting the use of diluted milk instead of TCA filtrate, but the lower values obtained with the filtrate were here attributed to the incomplete release by TCA of the casein-bound citric acid in milk. However, Evenhuis (1961) adhered to his opinion that the method of Marier & Boulet (1958) gave values about 10 % high, but he reported that their altered method appeared to give values only 2–3 % high and he maintained there was no evidence that the use of TCA filtrate gave low values. He concluded that further investigation was necessary before it could be said with certainty which method gave the correct value.

In previous publications by the present authors (White & Davies, 1958; Davies & White, 1959, 1960) values for the citric acid content of milk and milk sera were obtained by a modification of the method of Saffran & Denstedt (1948). This procedure, which involved the use of TCA filtrate (12 %, w/v, TCA), had an accuracy of about 100 % when assessed by the recovery of citric acid added to milk, and a precision of about ± 0·5 %. The advent of the two procedures of Marier & Boulet (1958, 1960) which, although appearing convenient, accurate and precise, had been adversely criticized by Davies (pers. comm.) and Evenhuis (1959, 1961), and the view of Marier & Boulet that values for the citric acid content of milk obtained by analysing milk-TCA filtrate are low, made it necessary to compare and reassess the accuracy of the various methods based on the pyridine–acetic anhydride–citric acid reaction for determining the citric acid content of milk and of milk sera.

EXPERIMENTAL

All the reagents used were of Analar grade (The British Drug Houses Ltd). For most of the work the pyridine and acetic anhydride were distilled, but it was found latterly that this treatment could be omitted even though the use of undistilled acetic anhydride led to a slight decrease in the sensitivity of the reaction. The pyridine and acetic anhydride were dispensed from 10 and 25 ml burettes respectively, which were fitted with 500 ml reservoir bottles and calcium chloride guard tubes. In the preparation of standard solutions for relating optical density to weight of citric acid, a freshly prepared standard stock solution of tri-sodium citrate (Marier & Boulet, 1959) was used.

Citric acid determinations were made on milk and milk ultrafiltrate and also on citrate-free milk and citrate-free milk ultrafiltrate to which known additions of citrate were made. The milk samples were either herd bulk milk or the milk of individual cows (all of the Ayrshire breed). To remove the fat, each milk, at room temperature, was centrifuged for 30 min at 1000*g*, a glass tube passed through the layer of fat and the separated milk collected by suction and used for analysis. All the analytical values for milk therefore apply to separated milk and not to whole milk. Milk ultrafiltrate was prepared from separated milk by the procedure described by Davies & White (1960). Citrate-free milk and citrate-free ultrafiltrate were prepared by passing separated milk and ultrafiltrate, under gravitational flow, through an anion exchange resin (Dowex, AG2–X8, 200–400 mesh, chloride form; V. A. Howe and Co. Ltd, London) previously washed with distilled water. In the preparation of citrate-free milk two columns of resin (16 × 1·3 cm), one above the other, were used. The first 5 ml of eluate from the upper column was not allowed to flow into the lower column, the first 15 ml of eluate from the lower column was also discarded, and the subsequent 25 ml of eluate collected (5 ml/h). This treatment always removed at least 99 % of the citric acid in the original milk, but when residual citric acid was found allowance was made for it. In the preparation of citrate-free ultrafiltrate only one column of resin (16 × 1·3 cm) was used. The first 20 ml of eluate was discarded and the subsequent 25 ml collected (15 ml/h). This treatment invariably removed all the citric acid from milk ultrafiltrate. Analysis of distilled water that had been passed through the washed resin and of this water containing the same concentration of chloride as resin-treated ultrafiltrate, each with and without added citrate, showed that the resin-treated milk and ultrafiltrate would be unlikely to be contaminated with any substance that interfered with citrate analysis. Citrate-free milk had a pH, total nitrogen content and total phosphorus content that were lower by 0·2 of a unit, 2·6 % and 50 %, respectively, than the values for the original milk; there was no change in lactose content.

To prepare TCA filtrate, milk, milk ultrafiltrate or the citrate-free equivalent of each was diluted in a volumetric flask with TCA solution to give a final TCA concentration of 12 % (w/v). The 24 % (w/v) TCA solution used for this purpose and also in the preparation of certain of the standard citrate solutions was filtered through a Whatman No. 42 paper before use. Allowance was made for the volume of the precipitate in the milk-TCA mixture on the basis that this volume averages 0·3 ml/10 ml of separated milk; with whole milk the corresponding volume would be 0·7 ml.

Description of methods

Method 1. *Modified method of Saffran & Denstedt*

Preparation of samples. Pipette 10·0 ml milk or ultrafiltrate into a 100 ml volumetric flask, add 40 ml water, mix gently and dilute to volume with 24 % (w/v) TCA solution. Shake the flask vigorously for a few seconds and after 30 min filter the mixture, using Whatman No. 40 paper for milk and No. 42 paper for ultrafiltrate, and collect the filtrate.

Preparation of standard citrate solutions. Dissolve 0·1913 g sodium citrate ($Na_3C_6H_5O_7 . 2H_2O$) in water and dilute to 100 ml in a volumetric flask. Pipette 0, 2·0,

3·0, 4·0 and 5·0 ml of this solution into 25 ml volumetric flasks, dilute to 12·5 ml with water and then to volume with 24 % (w/v) TCA solution. A volume of 1·0 ml of each of these solutions contains the equivalent of 0 (blank), 0·100, 0·150, 0·200 and 0·250 mg citric acid, respectively.

Procedure. Pipette 1·0 ml blank solution, standard citrate solutions and TCA filtrates into a series of test tubes (Pyrex, 15 × 1·5 cm, with glass stoppers) and place the tubes in random order; the maximum number of tubes in one group should be about twenty. Add 8·0 ml acetic anhydride to each tube, stopper tightly and swirl each tube to mix its contents. Place the tubes in a large, thermostatically controlled water bath at 60 °C for 10 min and then transfer them to a water bath at 20 °C for 5 min. Dry the tubes, add 1·0 ml pyridine to each, re-stopper tightly and return the tubes to the water bath at 60 °C, swirling each tube just before it is placed in the bath. After 45 min, transfer the tubes to the water bath at 20 °C and allow them to cool for 5 min. Dry the tubes and place them in a box for protection from light. Within the next 30 min measure the optical density of the standard and filtrate solutions relative to the blank using a wavelength of 428 mμ and a light path of 1 cm.

Method 2. *Method of Marier & Boulet using* TCA *filtrate*

Preparation of samples. Pipette 5·0 ml milk or ultrafiltrate into a 100 ml volumetric flask, add 45 ml water, mix gently and dilute to volume with 24 % (w/v) TCA solution. Shake the flask vigorously for a few seconds and after 30 min filter the mixture, using Whatman No. 40 paper for milk and No. 42 paper for ultrafiltrate, and collect the filtrate.

Preparation of standard citrate solutions. Dissolve 0·1913 g sodium citrate ($Na_3C_6H_5O_7 . 2H_2O$) in water and dilute to 200 ml in a volumetric flask. Pipette 0, 2·0, 3·0, 4·0 and 5·0 ml of this solution into 25 ml volumetric flasks, dilute to 12·5 ml with water and then to volume with 24 % (w/v) TCA solution. A volume of 1·0 ml of each of these solutions contains the equivalent of 0 (blank), 0·050, 0·075, 0·100 and 0·125 mg citric acid, respectively.

Procedure. Pipette 1·0 ml blank solution, standard citrate solutions and TCA filtrates into a series of test tubes (Pyrex, 15 × 1·5 cm, with glass stoppers) and place the tubes in random order; the maximum number of tubes in one group should be about twenty. Add 1·3 ml pyridine to each tube and swirl each tube to mix its contents. Add 5·7 ml acetic anhydride to the first tube, stopper, swirl the tube to mix its contents and immediately place it in a large, thermostatically controlled water bath at 32 °C. Do this with each tube in turn and leave the tubes in the water bath for 30 min from the time of insertion of the last tube. Dry the tubes and within the next 30 min measure the optical density of the standard and filtrate solutions relative to the blank using a wavelength of 428 mμ and a light path of 1 cm. The ambient temperature should not be less than 20 °C.

Method 3. *Method of Marier & Boulet using aqueous samples*

This method is the same as Method 2 except that distilled water alone is used for the blank and in the preparation of the milk (separated) and ultrafiltrate samples and of the standard citrate solutions.

Method 4. *Method of Marier & Boulet using aqueous–*NaOH *samples*

Preparation of samples. Pipette 10·0 ml milk (separated) or ultrafiltrate into a 100 ml volumetric flask and dilute to volume with water.

Preparation of standard citrate solutions. Dissolve 0·1913 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in water and dilute to 100 ml in a volumetric flask. Pipette 2·0, 3·0, 4·0 and 5·0 ml of this solution into 25 ml volumetric flasks and dilute to volume with water. A volume of 0·5 ml of each of these solutions contains the equivalent of 0·050, 0·075, 0·100 and 0·125 mg citric acid, respectively.

Procedure. Pipette 0·5 ml water (blank), standard citrate solutions and diluted samples into a series of test tubes (Pyrex, 15 × 1·5 cm, with glass stoppers): the maximum number of tubes in one group should be about twenty. Then pipette 0·5 ml water into the tube containing water (blank) and into the tubes containing the standard citrate solutions, and pipette 0·5 ml 0·4 N-NaOH into the tubes containing the samples. Place the tubes in random order and swirl each to mix its contents. Add 1·3 ml pyridine to each tube and then proceed as described in Method 2.

Method 5. *Modified method of Marier & Boulet using aqueous–*NaOH *samples*

This method is the same as Method 4 except that 0·5 ml 0·4 N-NaOH is added to the water (blank) and to the standard citrate solutions in place of 0·5 ml water, i.e. the blank, standard citrate solutions and diluted samples all receive an addition of sodium hydroxide.

The modified method of Saffran & Denstedt and the methods of Marier & Boulet described above differ only in small details from those originally published. The principal innovations in the method of Saffran & Denstedt are the use of a stronger TCA solution to ensure the complete precipitation of protein and a cooling treatment before adding the pyridine. In the methods of Marier & Boulet it was found that satisfactory measurements of optical density could be made if the ambient temperature was not less than 20 °C during the 30 min allowed for colour measurement. At lower air temperatures optical density tended to increase during the 30 min period and also as the period at 20 °C was extended beyond 30 min. Marier & Boulet (1958) state that the colour intensity remains constant for at least 30 min after the 30 min period allowed for colour development, either in the water bath at 32 °C or in air at a temperature of 22–29 °C; they recommend that if the gradient between bath and room temperature differs from these limits colour intensity should be determined within 10 min.

RESULTS AND DISCUSSION

Comparison of the modified method of Saffran & Denstedt (Method 1*) and the method of Marier & Boulet using* TCA *filtrate (Method* 2*)*

As no comparative study of citrate values obtained by the techniques of Saffran & Denstedt and Marier & Boulet appeared to have been made, the first step in this investigation was to compare Methods 1 and 2. Values for the citric acid content of twelve milks and four ultrafiltrates obtained by the two methods are listed in Table 1. Method 1 gave values that were, on average, 96 and 97 % of those obtained by Method 2 for milk and ultrafiltrate, respectively. The standard error of the mean of

duplicate determinations, calculated from the data used to obtain the values in Table 1, was 1·1 and 2·1 mg of citric acid per 100 ml of milk or ultrafiltrate for Methods 1 and 2, respectively.

Table 1. *Values for the citric acid content of milk and milk ultrafiltrate obtained by (a) the method of Marier & Boulet using TCA filtrate (Method 2) and (b) the modified method of Saffran & Denstedt (Method 1); each value is the mean of duplicate determinations*

	Citric acid, mg/100 ml			
Milk	(*a*)	(*b*)	(*b* − *a*)	(*b*/*a* × 100)
1	200	188	−12	94·0
2	168	163	−5	97·0
3	152	142	−10	93·4
4	111	104	−7	93·7
5	188	176	−12	93·6
6	169	166	−3	98·2
7	178	175	−3	98·3
8	233	229	−4	98·3
9	197	190	−7	96·4
10	186	181	−5	97·3
11	150	146	−4	97·3
12	156	149	−7	95·5
			Mean	96·1
Ultrafiltrate				
a	192	188	−4	97·9
b	167	157	−10	94·0
c	146	144	−2	98·6
d	154	150	−4	97·4
			Mean	97·0

The accuracy of both methods over a wide range of citric acid concentration, when assessed by the recovery of citrate added to milk and diluted milk, was found to be approximately 100 %. This was surprising in view of the comparative values given in Table 1 and so the accuracy of both methods was further examined by recovery experiments using citrate-free ultrafiltrate. In these experiments, various standard citrate solutions were added to 100 ml volumetric flasks, each containing the volume of citrate-free ultrafiltrate appropriate to the method, i.e. 10 ml and 5 ml for Methods 1 and 2, respectively, and the contents of the flasks diluted to volume with TCA solution to a final TCA concentration of 12 % (w/v). The mixtures were then filtered through Whatman No. 42 paper and the filtrates analysed. At each level of citrate, Method 1 underestimated the citric acid by about 8 mg per 100 ml of citrate solution (Table 2) whereas Method 2 gave satisfactory recoveries (Table 3). The percentage accuracy of Method 1 (Table 2) at the levels of citric acid normally present in milk or ultrafiltrate, i.e. 150–250 mg per 100 ml, is similar to the values in the last column of Table 1. It would appear therefore that citrate values for milk obtained by Method 1 will be low to at least the same extent as those for ultrafiltrate. That the trace of protein in citrate-free ultrafiltrate was not an interfering factor was evident from the fact that recoveries by Method 1 of citrate added to TCA filtrate prepared from citrate-free ultrafiltrate were still only 95–97 %.

It seemed that interference from some ultrafilterable constituent or constituents of milk that passed through the anion exchange resin was responsible for the small

negative error in the recovery values obtained by Method 1. Confirmation of the error, by magnifying it, was obtained from recovery experiments in which a standard citrate solution similar in citrate content to milk of average composition was added to different volumes of citrate-free ultrafiltrate and the TCA filtrates analysed by

Table 2. *Values for citric acid obtained by analysing standard citrate solutions plus 10 ml of citrate-free milk ultrafiltrate by the modified method of Saffran & Denstedt (Method 1); each determined value is the mean of six obtained with three ultrafiltrates*

	Citric acid, mg/100 ml citrate solution			
	Theoretical (a)	Determined (b)	Difference ($b-a$)	Accuracy ($b/a \times 100$)
	0	0	0	—
	50	42	−8	84·0
	100	92	−8	92·0
*	150	142	−8	94·7
*	200	193	−7	96·5
*	250	242	−8	96·8
	300	290	−10	96·7

* Values in the range normally found in milk.

Table 3. *Values for citric acid obtained by analysing standard citrate solutions plus 5 ml of citrate-free milk ultrafiltrate by the method of Marier & Boulet using TCA filtrate (Method 2); each determined value is the mean of four obtained with two ultrafiltrates*

Citric acid, mg/100 ml citrate solution			
Theoretical (a)	Determined (b)	Difference ($b-a$)	Accuracy ($b/a \times 100$)
0	0	0	—
50	51	1	102·0
100	101	1	101·0
150	152	2	101·3
200	199	−1	99·5
250	250	0	100·0
300	297	−3	99·0
350	348	−2	99·4
		Mean	100·3

Table 4. *Values for citric acid obtained by analysing a standard citrate solution (similar in citrate content to milk of average composition) plus different volumes of citrate-free milk ultrafiltrate by the modified method of Saffran & Denstedt (Method 1); each determined value is the mean of six obtained with three ultrafiltrates*

Vol. citrate-free ultrafiltrate (ml)	Citric acid, mg/100 ml citrate solution			
	Theoretical (a)	Determined (b)	Difference ($b-a$)	Accuracy ($b/a \times 100$)
0	200	200	0	100·0
10*	200	193	−7	**96·5**
20	200	189	−11	94·5
30	200	185	−15	92·5
40	200	181	−19	90·5

* Normal sample volume.

both methods. The values obtained by Method 1 (Table 4) show that the recoveries decreased with the volume of citrate-free ultrafiltrate used. The values obtained by Method 2 (Table 5) confirmed that the recovery with the normal sample volume was satisfactory but show that, as in Method 1 (Table 4), when the volume of citrate-free ultrafiltrate is increased, an increasing level of interference caused a decreasing recovery of citric acid.

Table 5. *Values for citric acid obtained by analysing a standard citrate solution (similar in citrate content to milk of average composition) plus different volumes of citrate-free milk ultrafiltrate by the method of Marier & Boulet using TCA filtrate (Method 2); each determined value is the mean of six obtained with three ultrafiltrates*

Vol. citrate-free ultrafiltrate (ml)	Citric acid, mg/100 ml citrate solution			
	Theoretical (a)	Determined (b)	Difference ($b-a$)	Accuracy ($b/a \times 100$)
0	200	201	1	100·5
5*	200	198	−2	99·0
10	200	196	−4	98·0
15	200	193	−7	96·5
20	200	191	−9	95·5
25	200	188	−12	94·0

* Normal sample volume.

From the values in Tables 3 and 5 it seems reasonable to conclude that when applied to ultrafiltrate the method of Marier & Boulet using TCA filtrate has an accuracy in the range 99–102 %, whereas the values in Tables 2 and 4 indicate that the corresponding accuracy of the modified method of Saffran & Denstedt is of the order of 95–97 %. The fact that the modified method of Saffran & Denstedt appeared to have a satisfactory accuracy when judged by the recovery of the citrate added to milk and yet in reality has an accuracy not exceeding 95–97 %, emphasizes that recovery tests of this nature do not give an unequivocal measure of accuracy. Because of the inherent error, albeit small, in the modified method of Saffran & Denstedt (Method 1) no further use was made of this method in the present investigation.

Comparison of the method of Marier & Boulet using TCA *filtrate (Method* 2*) and the method of Marier & Boulet using aqueous samples (Method* 3*)*

Marier & Boulet (1958) preferred the use of diluted fat-free milk (Method 3) to that of milk-TCA filtrate (Method 2) because the latter was believed to give values for the citric acid content of milk that were about 7 % low. However, as already mentioned, there is the possibility that the difference in values obtained with the two milk preparations arises from high values with diluted milk. In an attempt to resolve these conflicting opinions, values for the citric acid content of twelve milks and three ultrafiltrates were obtained by Methods 2 and 3 (Table 6). These results show that the methods gave values that were in agreement with ultrafiltrate but with milk the values obtained with Method 3 exceeded those obtained with Method 2 by an average of about 34 %. The reason for the large discrepancy in the milk values was readily ascertained: it was noticed that in every instance the reaction mixture containing

diluted milk was opalescent or even turbid and there seems little doubt that this opalescence or turbidity caused a gross over-estimate of the intensity of the yellow colour. These results agree with those of Evenhuis (1959, 1961) but the degree of interference by the opalescence was more serious in the present work than that encountered by Evenhuis. It is difficult to reconcile these findings with the reports of Marier & Boulet (1958, 1960) and Marier *et al.* (1961) that the use of diluted milk does not result in opalescent solutions, or if it does, the effect is insignificant. It can be concluded only that some difference in the reagents, possibly in the acetic acid content of the acetic anhydride used, or some difference in the temperature reached by the reaction mixtures during colour development, possibly from a small procedural difference, is responsible for the divergent findings. Because in the present investigation the method of Marier & Boulet using aqueous samples (Method 3) gave values for the citric acid content of milk which were far too high, no further investigation of this method was carried out.

Table 6. *Values for the citric acid content of milk and milk ultrafiltrate obtained by (a) the method of Marier & Boulet using TCA filtrate (Method 2) and (b) the method of Marier & Boulet using aqueous samples (Method 3); each value is the mean of three*

	Citric acid, mg/100 ml			
Milk	(*a*)	(*b*)	(*b* − *a*)	(*b*/*a* × 100)
1	162	238	76	146·9
2	187	251	64	134·2
3	175	223	48	127·4
4	192	258	66	134·4
5	193	256	63	132·6
6	211	285	74	135·1
7	171	231	60	135·1
8	168	230	62	136·9
9	167	213	46	127·5
10	194	260	66	134·0
11	170	227	57	133·5
12	187	253	66	135·3
			Mean	134·4
Ultrafiltrate				
a	204	202	−2	99·0
b	166	164	−2	98·8
c	186	190	4	102·2
			Mean	100·0

*Comparison of the method of Marier & Boulet using TCA filtrate (Method 2), the method of Marier & Boulet using aqueous–*NaOH *samples (Method 4) and the modified method of Marier & Boulet using aqueous–*NaOH *samples (Method 5)*

Marier & Boulet (1960) altered their method for the determination of citric acid in milk (Method 3) by making an addition of a small amount of sodium hydroxide to the diluted milk samples to obviate any risk of opalescence (Method 4). They did not make a corresponding addition to the blank and to the standard solutions, because they believed that the sodium hydroxide did not interfere significantly with the reaction (Marier & Boulet, 1958, 1960; Marier, pers. comm.). Values for the citric acid content of milk obtained by Methods 2 and 4 and also by Method 5, which is the same

as Method 4 except that sodium hydroxide is added to the blank and to the standard solutions as well as to the diluted milks, are given in Table 7. The results show that Method 4 gave values that are on average about 7 % higher than those obtained with Method 2, which is in agreement with the view of Marier & Boulet (1958, 1960) and Marier *et al.* (1961) that the use of TCA filtrates gives values 4–10 % lower than when aqueous–NaOH samples are used. However, the results show also that Method 5 gave values higher on average by only about 4 % than those obtained with Method 2. In these comparisons none of the reaction mixtures containing diluted milk and sodium hydroxide showed obvious signs of opalescence or turbidity.

Table 7. *Values for the citric acid content of milk obtained by* (*a*) *the method of Marier & Boulet using TCA filtrate* (*Method* 2), (*b*) *the method of Marier & Boulet using aqueous–*NAOH *samples* (*Method* 4) *and* (*c*) *the modified method of Marier & Boulet using aqueous–*NaOH *samples* (*Method* 5)*; each value is the mean of duplicate determinations*

	Citric acid, mg/100 ml				
Milk	(*a*)	(*b*)	(*c*)	(*b*/*a* × 100)	(*c*/*a* × 100)
1	183	198	192	108·2	104·9
2	197	206	201	104·6	102·0
3	160	174	169	108·8	105·6
4	182	196	190	107·7	104·4
5	233	—	241	—	103·4
6	208	—	213	—	102·4
7	198	—	204	—	103·0
8	124	—	134	—	108·1
9	207	—	218	—	105·3
10	195	—	209	—	107·2
11	252	—	261	—	103·6
12	174	—	180	—	103·4
				Mean 107·3	104·4

The probable reason for the better agreement between values obtained with the method of Marier & Boulet using TCA filtrate (Method 2) and the modified method of Marier & Boulet using aqueous–NaOH samples (Method 5) was apparent when the optical densities of the standard citrate reaction mixtures in Methods 2, 4 and 5 were compared. It was found that the optical densities of the corresponding standard citrate reaction mixtures in Methods 2 and 4 were identical whereas those in Method 5 were about 2 % higher, i.e. the colour intensity in TCA or aqueous standard citrate reaction mixtures was the same but the presence of sodium hydroxide caused colour enhancement. As this finding conflicted with the statement of Marier & Boulet (1960) that up to 0·5 m-equiv. of NaOH (equivalent to 0·5 ml of N-NaOH) could be 'tolerated per tube' further comparisons were made of the optical densities obtained with aqueous standards and with aqueous standards to which 0·5 ml of 0·4 N- or 0·8 N-NaOH had been added. The results (Table 8) show that the presence of 0·5 ml of 0·4 N-NaOH and 0·5 ml of 0·8 N-NaOH increased the optical densities of the citrate standards, at all the levels of citric acid, by about 2 and 24 %, respectively. With the standards containing the larger amount of sodium hydroxide the tubes required an additional swirling, about 1 min after they had been placed in the water bath, to ensure that homogeneous solutions were obtained; to maintain uniformity in this

comparative study all the standards received the additional mixing although this was unnecessary with aqueous standards and also probably with those containing the smaller amount of sodium hydroxide. Without the additional mixing, the standards containing the larger amount of sodium hydroxide usually gave very poor replication of optical density, but when duplicates agreed reasonably well the optical densities were only 5% higher than those for the aqueous standards, whereas those for

Table 8. *Optical densities of standard citrate reaction mixtures containing* (*a*) 0·5 *ml citrate solution plus* 0·5 *ml water*, (*b*) 0·5 *ml citrate solution plus* 0·5 *ml* 0·4 N-NaOH *and* (*c*) 0·5 *ml citrate solution plus* 0·5 *ml* 0·8 N-NaOH*; each value is the mean of eight replicate determinations*

Citric acid (mg)	Optical density, 428 mμ, 1 cm (*a*)	(*b*)	(*c*)	(*b*/*a* × 100)	(*c*/*a* × 100)
0·050	0·134	0·136	0·167	101·5	124·4
0·075	0·190	0·193	0·236	101·6	124·3
0·100	0·242	0·246	0·297	101·7	122·7
0·125	0·289	0·295	0·360	102·1	124·4
0·150	0·335	0·342	0·412	102·1	123·1
0·200	0·419	0·426	0·515	101·7	122·9
0·250	0·503	0·514	0·625	102·2	124·2
			Mean	101·8	123·7

Table 9. *Values for the citric acid content of milk obtained by the modified method of Marier & Boulet using aqueous–*NaOH *samples* (*Method* 5), (*a*) *with the addition of* 0·4 N-NaOH *and* (*b*) *with the addition of* 0·8 N-NaOH*; each value is the mean of duplicate determinations*

Milk	Citric acid, mg/100 ml (*a*)	(*b*)	(*b* − *a*)	(*b*/*a* × 100)
1	180	144	−36	80·0
2	216	175	−41	81·0
3	211	167	−44	79·1
4	247	192	−55	77·7
5	232	186	−46	80·2
6	216	169	−47	78·2
			Mean	79·4

the standards containing the smaller amount were again 2% higher. These increments of approximately 2% and 5% are consistent with data supplied by Marier (pers. comm.) on the effect of various concentrations of sodium hydroxide on colour intensity. However, even the small increase in optical density when the smaller amount of sodium hydroxide is incorporated in the standards cannot be regarded as insignificant, for to ignore it, as is done in Method 4 by simply using aqueous standards, will incur an error from this source alone of about +3% in values for citric acid. From these results it was apparent that when sodium hydroxide is added to the samples being analysed it must also be added to the blank and to the standard solutions.

Even though the modified method of Marier & Boulet using aqueous–NaOH samples (Method 5) meets the requirement of having sodium hydroxide in the blank and in the standard solutions as well as in the diluted milk samples, it gave

values for the citric acid content of milk that varied with the strength of sodium hydroxide solution used. This is illustrated by the values in Table 9; with the larger amount of sodium hydroxide the values for citric acid were on average only about 79 % of those with the smaller amount. The reason for this result was that the optical densities of the milk samples, after colour development, were the same whether 0·4 or 0·8 N-NaOH was used, whereas those of the standard solutions with 0·8 N-NaOH were about 24 % higher than those of the corresponding standards with 0·4 N-NaOH (cf. Table 8). This rather curious finding may be a consequence of a difference in the alkalinity of the corresponding standard and milk reaction mixtures in the early stages of colour development: it was noticed that diluted milks containing 0·8 N-NaOH gave homogeneous reaction mixtures more rapidly than did standard citrate solutions containing 0·8 N-NaOH. The addition of up to 0·5 m-equiv. of NaOH (equivalent to 0·5 ml of N-NaOH) to the sample of diluted milk, which Marier & Boulet (1960) say is permissible and sometimes necessary to avoid opalescence, therefore seems fraught with difficulties and the possibility of using more sodium hydroxide than the 0·5 ml of 0·4 N solution used in Method 5 was abandoned.

*Validity of citric acid values obtained by the method of Marier & Boulet using TCA filtrate (Method 2) and the modified method of Marier & Boulet using aqueous–*NaOH *samples (Method 5)*

It has been shown that values for the citric acid content of milk obtained by Method 5 exceed those obtained by Method 2 by an average of about 4 % (Table 7). On the other hand, the results in Table 10 show that these methods gave values for the citric acid content of milk ultrafiltrate that are very similar: since Method 2 has been shown to give correct values for the citric acid content of ultrafiltrate (Table 3) it follows that Method 5 also gives correct values when applied to ultrafiltrate. It

Table 10. *Values for the citric acid content of milk ultrafiltrate obtained by (a) the method of Marier & Boulet using TCA filtrate (Method 2) and (b) the modified method of Marier & Boulet using aqueous–*NaOH *samples (Method 5); each value is the mean of triplicate determinations*

	Citric acid, mg/100 ml			
Ultrafiltrate	(*a*)	(*b*)	(*b* − *a*)	(*b*/*a* × 100)
1	172	170	−2	98·8
2	163	160	−3	98·2
3	186	185	−1	99·5
4	212	213	1	100·5
5	170	165	−5	97·1
6	176	176	0	100·0
7	192	185	−7	96·4
8	170	169	−1	99·4
9	176	178	2	101·1
			Mean	99·0

remained therefore to find why Methods 2 and 5 gave different values for citric acid when applied to milk and to decide whether either method gave the correct value. As previously mentioned, there are conflicting views on the reason for the difference in the values obtained by these two methods when applied to milk. It is possible that

TCA does not bring into solution all the casein-bound citric acid, this being the latest view of Marier *et al.* (1961), and consequently values obtained by Method 2 may be low. It is also possible that with Method 5, despite the addition of sodium hydroxide both to standards and samples and the absence of any obvious opalescence, the values obtained may still be high. These two possibilities were therefore investigated.

Table 11. *Values for citric acid obtained by analysing standard citrate solutions plus 5 ml of citrate-free milk by the method of Marier & Boulet using TCA filtrate (Method 2); each determined value is the mean of four obtained with two milks*

Citric acid, mg/100 ml citrate solution			
Theoretical (*a*)	Determined (*b*)	Difference (*b* − *a*)	Accuracy (*b*/*a* × 100)
0	0	0	—
100	99	−1	99·0
150	150	0	100·0
200	198	−2	99·0
250	247	−3	98·8
		Mean	99·2

Table 12. *Values for citric acid obtained by analysing a standard citrate solution (similar in citrate content to milk of average composition) plus different volumes of citrate-free milk by the method of Marier & Boulet using TCA filtrate (Method 2); each determined value is the mean of eight obtained with two milks*

Vol. citrate-free milk (ml)	Citric acid, mg/100 ml citrate solution			
	Theoretical (*a*)	Determined (*b*)	Difference (*b* − *a*)	Accuracy (*b*/*a* × 100)
0	200	200	0	100·0
5*	200	198	−2	99·0
10	200	195	−5	97·5
15	200	194	−6	97·0
20	200	191	−9	95·5

* Normal sample volume.

Analysis by Method 2 of milk-TCA filtrate prepared in the usual way (p. 174) and of milk-TCA filtrate prepared by the dropwise addition of the 24% (w/v) TCA solution so that the protein coagulated slowly to form very small clots, followed by prolonged shaking of the mixture before filtration, gave the same value for the citric acid content of the milk (cf. Evenhuis, 1961). This, together with the fact that a milk-TCA filtrate (12%, w/v, TCA) contains all the calcium and inorganic phosphorus originally attached to the casein, both of which appear to be closely associated with the casein-bound citric acid (Pyne & McGann, 1960), suggests that it is unlikely that TCA does not bring into solution all the casein-bound citric acid. Recovery experiments with citrate-free milk containing added citrate provided further evidence of the suitability of TCA filtrate for determining the citric acid content of milk. In these experiments, various standard citrate solutions were added to 100 ml volumetric flasks each containing 5 ml of citrate-free milk, the contents of the flasks diluted to volume with TCA solution to give a final TCA concentration of 12% (w/v)

and the mixtures filtered. The results from the analysis of the filtrates (Table 11) make it probable that the method of Marier & Boulet using TCA-filtrate (Method 2), when applied to milk, has an accuracy of about 99 %. Further recovery experiments in which a standard citrate solution similar in citrate content to milk of average composition was added to different volumes of citrate-free milk (Table 12) showed that, as with citrate-free ultrafiltrate (Table 5), the recoveries by Method 2 decrease as the volume of citrate-free sample exceeds 5 ml. However, recovery experiments with Method 5 (Table 13), while confirming that this method is reasonably accurate when applied to ultrafiltrate (recoveries of 97–99 %), revealed that it overestimated citrate added to citrate-free milk by 3–4 %. It would appear therefore from all the evidence presented that the method of Marier & Boulet using TCA filtrate (Method 2) gives correct values for the citric acid content of both milk and ultrafiltrate whereas the modified method of Marier & Boulet using aqueous–NaOH samples (Method 5), although giving a correct value for the citric acid content of ultrafiltrate, gives a high value with milk. Further evidence supporting this criticism of Method 5 will now be given.

Table 13. *Values for citric acid obtained by analysing a standard citrate solution (similar in citrate content to milk of average composition) plus 10 ml of citrate-free milk ultrafiltrate or of citrate-free milk by the modified method of Marier & Boulet using aqueous–*NaOH *samples (Method 5); each determined value is the mean of four replicate determinations*

	Citric acid, mg/100 ml citrate solution			
Ultrafiltrate	Theoretical (*a*)	Determined (*b*)	Difference (*b* − *a*)	Accuracy (*b*/*a* × 100)
a	200	197	−3	98·5
b	200	194	−6	97·0
c	200	198	−2	99·0
			Mean	98·2
Milk				
1	200	206	6	103·0
2	200	206	6	103·0
3	200	208	8	104·0
			Mean	103·3

The presence of protein in the reaction mixtures used in Method 5 seemed the most likely cause of any positive error. However, with this method definite opalescence was not visible in the coloured solutions obtained with milk, or citrate-free milk to which citrate had been added. But in reaction mixtures containing citrate-free milk without added citrate there was a slight, definite opalescence no doubt now visible because of the absence of any developed yellow coloration. As it seems unlikely that the resin treatment used in the preparation of the citrate-free milk would produce a milk causing any more opalescence than an untreated milk, it is highly probable that the reaction mixtures used in Method 5 always had a trace of opalescence but that when citric acid is present the yellow colour which develops masks the opalescence.

Indirect evidence of the presence of opalescence in the milk sample solutions used in Method 5 is furnished by the typical absorption curves in Figs. 1 and 2. Those in Fig. 1 show that milk and standard citrate solution treated as in the method of

Marier & Boulet using TCA filtrate (Method 2) gave curves of the same shape and without absorption at wavelengths greater than 850 mμ; with citrate-free milk there was no significant absorption over the whole range of wavelengths covered. The curve forming the top part of Fig. 1 is a plot of the difference between the curves for the milk and the standard citrate solution and is typical of that for a solution containing a small amount of citric acid. The curves in Fig. 2 were obtained from milk, citrate-free milk and standard citrate solution treated as in the modified method of Marier & Boulet using aqueous–NaOH samples (Method 5). The curve for the standard citrate solution has exactly the same shape as the curves for milk and standard

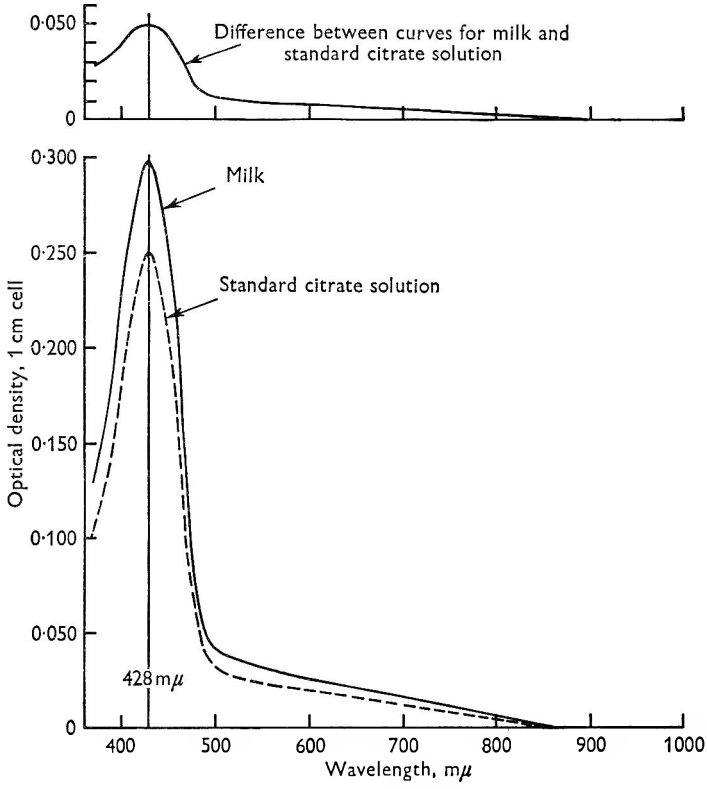

Fig. 1. Typical absorption curves obtained with milk and standard citrate solution (0·100 mg citric acid) by the method of Marier & Boulet using TCA filtrate (Method 2) and also the plot of the difference between the curves for the milk and the standard solution.

citrate solution in Fig. 1, with a maximum at 428 mμ and no absorption at wavelengths greater than 850 mμ. That for the milk also has an absorption maximum at 428 mμ but indicates some absorption at wavelengths greater than 850 mμ. The curve for the citrate-free milk is very different in shape, however: there is no maximum at 428 mμ, absorption increases steadily with decreasing wavelength and, as with the milk, there is some absorption at wavelengths greater than 850 mμ. As the solution containing citrate-free milk showed a faint opalescence but no trace of yellow colour, this abnormally shaped absorption curve can be fairly safely attributed to the opalescence. The curve forming the top part of Fig. 2 is a plot of the difference

between the curves for the milk and the standard citrate solution. It is atypical of that for a citrate solution. However, it can be seen that a curve of this type can be obtained by the combination of the bottom curve in Fig. 2 and the top curve in Fig. 1, i.e. it is characteristic of a slightly opalescent solution containing a small amount of citric acid. It was therefore concluded that the presence of opalescence in the milk reaction mixtures obtained with Method 5 in this investigation was the reason for this method giving values for the citric acid content of milk about 4 % higher on average than those given by Method 2 (Table 7), and for the over-estimation to a similar extent of citrate added to citrate-free milk (Table 13).

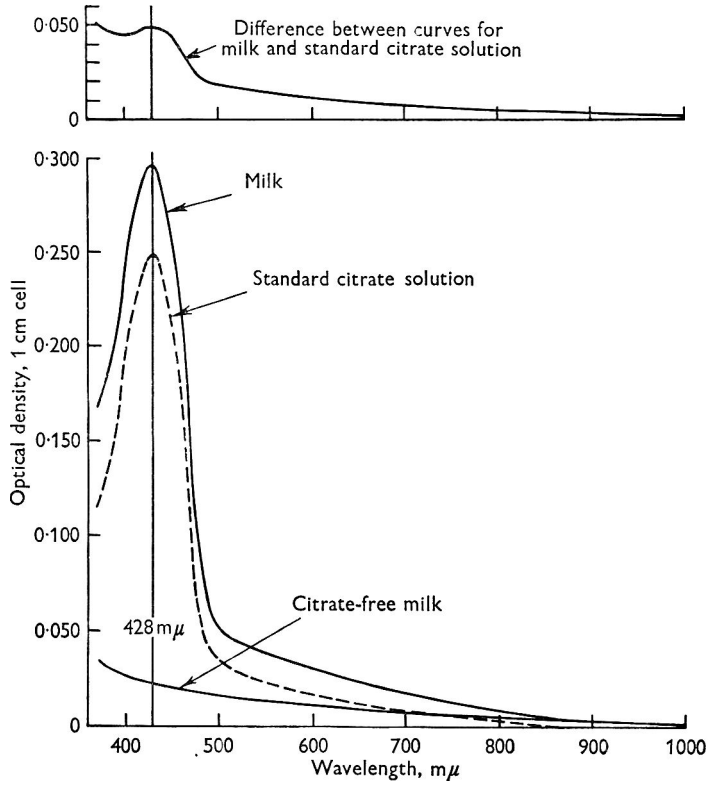

Fig. 2. Typical absorption curves obtained with milk, citrate-free milk and standard citrate solution (0·100 mg citric acid) by the modified method of Marier & Boulet using aqueous–NaOH samples (Method 5) and also the plot of the difference between the curves for the milk and the standard solution.

In view of this conclusion, it would be expected that if allowance were made for the opalescence occurring in Method 5 values for citric acid obtained by this method and by Method 2 should come into agreement. To test this hypothesis, a series of milks and the corresponding citrate-free milks were analysed by the two methods. The results (Table 14) show that by correcting for opalescence in Method 5, i.e. by subtracting the optical density obtained with the citrate-free milks from the optical density obtained with the milks, the respective values for the citric acid content of the milks were in good agreement. With no correction for opalescence, Method 5, as expected, gave higher values than Method 2.

Additional evidence supporting the above view was obtained by analysing milks by Methods 2 and 5 but using in the latter double the usual volumes of reactants, i.e. using 1 ml diluted milk, 1 ml 0·4 N-NaOH, 2·6 ml pyridine and 11·4 ml acetic anhydride, in tubes 15 × 2·5 cm instead of 15 × 1·5 cm. The results (Table 15) show that these methods gave virtually identical values, a finding attributable to the absence of opalescence when double volumes were used in Method 5. The absence of opalescence probably resulted from the fact that the maximum temperature reached by the reaction mixtures was about 50 °C, whereas with the normal volumes

Table 14. *Values for the citric acid content of milk obtained by* (*a*) *the method of Marier & Boulet using TCA filtrate* (*Method* 2), (*b*) *the modified method of Marier & Boulet using aqueous*–NaOH *samples* (*Method* 5) *and* (*c*) *Method* 5 *with allowance for opalescence: each value is the mean of triplicate determinations*

	Citric acid, mg/100 ml				
Milk	(*a*)	(*b*)	(*c*)	(*b*/*a* × 100)	(*c*/*a* × 100)
1	217	226	216	104·1	99·5
2	181	188	177	103·9	97·8
3	159	170	160	106·9	100·6
4	188	200	189	106·4	100·5
5	206	218	203	105·8	98·5
6	196	213	195	108·7	99·5
			Mean	106·0	99·4

Table 15. *Values for the citric acid content of milk obtained by* (*a*) *the method of Marier & Boulet using TCA filtrate* (*Method* 2) *and* (*b*) *the modified method of Marier & Boulet using aqueous*–NaOH *samples* (*Method* 5) *with double volumes of reactants; each value is the mean of triplicate determinations*

	Citric acid, mg/100 ml			
Milk*	(*a*)	(*b*)	(*b* − *a*)	(*b*/*a* × 100)
1	162	164	2	101·2
2	187	189	2	101·1
3	175	175	0	100·0
4	192	196	4	102·1
5	193	190	−3	98·4
6	211	211	0	100·0
7	171	169	−2	98·8
8	168	170	2	101·2
9	167	167	0	100·0
10	194	196	2	101·0
11	170	170	0	100·0
12	187	189	2	101·1
			Mean	100·4

* Same series as in Table 6.

of reactants the maximum temperature was 41–44 °C (cf. Hartford, 1962) because of a more rapid dissipation of heat from the smaller volume of liquid in a narrower tube. The higher temperature was apparently sufficient to reduce the opalescence, by causing the dispersion or solution of the material causing opalescence (presumably protein), by such an extent that it no longer interfered with the measurement of colour intensity. These results support the suggestion already made that differences in the

maximum temperature reached by reaction mixtures containing diluted milk may be responsible for some of the discrepancies in values for citric acid obtained by Marier & Boulet, Evenhuis and the present authors: one cause of such differences could be the influence of different ambient air temperatures on the initial temperature of the reactants.

CONCLUSIONS

The modified method of Saffran & Denstedt used in the present investigation (Method 1) underestimated the citric acid content of both milk and milk ultrafiltrate by 3–5 %. This small negative error appeared to be caused by interference from some soluble milk constituent or constituents.

The method of Marier & Boulet (1958) in which the sample taken for analysis is 1 ml of diluted separated milk or diluted milk ultrafiltrate, i.e. Method 3, gave an accurate value for the citric acid content of ultrafiltrate but a value about 34 % high for the citric acid content of milk. This error was caused by opalescence in the solutions used for colour measurement: a similar fault in this method, but not of such magnitude, has been found also by Evenhuis (1959, 1961). The method of Marier & Boulet (1960) in which the sample analysed is 0·5 ml of diluted separated milk or diluted milk ultrafiltrate, plus in each instance 0·5 ml of 0·4 N-NaOH, and in which aqueous standard citrate solutions are used, i.e. Method 4, gave a value on average about 7 % high for the citric acid content of milk. About three-sevenths of this error was caused by the omission of sodium hydroxide from the standards and about four-sevenths by a trace of opalescence in the sample solutions. With Method 4, the omission of sodium hydroxide from the standards would likewise cause an over-estimate of the citric acid content of ultrafiltrate by about 3 %. By modifying the method of Marier & Boulet (1960) to include sodium hydroxide in the blank and standards, i.e. Method 5, an accurate value for the citric acid content of milk ultrafiltrate could be obtained, but with milk the opalescence again caused an error of about +4 % in the value for citric acid. The latter finding supports the view of Evenhuis (1961) that Method 5 gives a value for the citric acid content of milk which is slightly high.

However, the method of Marier & Boulet using TCA filtrate (Method 2), in which the 1 ml sample analysed is taken from the filtrate prepared after the dilution of 5 ml of milk (whole or separated) or milk ultrafiltrate to 100 ml with TCA solution to give a final TCA concentration of 12 % (w/v), gave values for citric acid with an accuracy of 99–100 %. The standard error of the mean of duplicate determinations by this method is about 2 mg per 100 ml of milk or ultrafiltrate, which with samples of average citric acid content corresponds to a precision of about ±1 %. Method 2, described on p. 174, is therefore recommended for the determination of citric acid in milk and milk sera.

Marier & Boulet (1958, 1960) and Marier *et al.* (1961) have questioned the suitability of acid filtrates for the determination of the citric acid content of milk, and describe experiments which purport to show that the use of TCA filtrate gives values about 7 % (4–10 %) low. In the first instances the 'error' was attributed to adsorption of citric acid on the precipitated protein and in the second to incomplete release of casein-bound citrate. In view of the present findings these experiments would appear to require a different interpretation. That washed casein (or washed milk protein),

when suspended in a citrate solution of pH 4·5–4·6, appears capable of adsorbing citrate cannot be taken as evidence that the same adsorption occurs in the preparation of a TCA-milk filtrate; indeed as Marier *et al.* (1961) show, when such a suspension contains other milk constituents and/or is treated with TCA (pH < 1), adsorption does not occur. Also, the fact that casein precipitated from milk by adjusting the pH to 4·5–4·6 with hydrochloric acid (a procedure they appear to regard as achieving the same object as adjusting the pH to < 1 with a TCA solution) requires very thorough washing to remove all traces of citric acid, calcium and lactose is, in the opinion of the present authors, simply an indication of the difficulty of removing, not bound constituents, but the constituents of the contaminating mother liquid. The evidence obtained in the present investigation makes it highly improbable that any significant amount of citric acid remains bound to casein in the presence of a 12 % (w/v) solution of TCA; and there is ample evidence, recently confirmed (Davies & White, 1962), that under the same conditions all the calcium is in solution, and in all probability the same status exists after the pH of milk is reduced to 4·5–4·6 (Davies & White, 1960). Likewise, it would appear from the work of Grimbleby (1956) to be unlikely that any lactose remains bound to protein at the isoelectric point of casein or in the presence of relatively large concentrations of TCA. It is still not possible, however, to reconcile the results obtained in the present investigation with those of Marier *et al.* (1961) which indicate that when synthetic milk solutions are treated with TCA (final TCA concentration 12 %, w/v), the TCA filtrates are deficient in citric acid. In the similar experiments described here, TCA filtrates, prepared from citrate-free milk to which citrate had been added, contained on average 99·2 % of the theoretical amount of citric acid (Table 11).

The authors thank Miss H. K. Murray and Miss V. D. Young for technical assistance.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 191

Some aspects of machine milking rate

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(*Received* 15 *October* 1962)

Summary. A study was made of the machine milking rates of a sample of Jersey cows comprising daughter–dam pair groups, obtained from thirteen New Zealand herds during mid-lactation.

Twenty-one per cent of the variation in maximum rate of flow occurred within herds and the remainder between herds. Differences in the type of preparation of cows for milking did not have a significant effect on the average milking rates for herds. The variance in milking time between cows within poorly prepared herds was greater, however, than that in the better prepared herds.

Heritability estimates for maximum rate of flow, derived from the regression of daughters' records on dams' records, were found to be of the order of 80 %.

Apart from providing useful references to earlier work, Dodd (1953) described the variation in milking rates within a herd and also the influence of several factors, including stage of lactation and lactation age, on the normal milking rate of individual cows. Data on milking rate provided by the New Zealand Dairy Board (1950) indicated marked differences between the daughters of different bulls within herds, and also between the artificially bred daughters by the same sire in different herds.

Between-herd differences in milking rate are probably largely due to environmental factors rather than genetic differences (Brumby, 1961) but this has not been established with any accuracy.

Estimates for the 'heritability' of maximum rate of flow, derived from the milking rate records of monozygotic twins, are in good agreement (0·72–0·85) but have, however, been obtained from small groups of cows in single herds (Brumby, 1956; Donald, 1960).

This paper presents further information on the variation in milking rates within and between commercial herds and on the influence of the technique of stimulation of milk ejection on the differences between herds. The heritability of the milking rate characteristic was also estimated.

Experimental methods

Source of the data

During the period from December to February milk flow rates were obtained from dams and daughters in thirteen Jersey herds on farms situated in the South Auckland, Waikato, and Manawatu areas. One milking rate record was obtained at a morning milking from each of 249 different animals comprising 150 daughter–dam pairs. Some

of these were 'three generation groups' of grandam, dam, and daughter, and some of the dams had more than one daughter. The farms were chosen because of their owners' association with the College, but there was no reason to suppose that the herds were not representative.

Apparatus and measures of milking rate

The apparatus used for measuring milking rate was similar to that designed by Whittlestone and described by Brumby (1956).

The technique of preparation for milking was common for all animals within a herd but was different between herds. Stimulation was, as far as possible, that to which cows were accustomed, so as to avoid the possibility of a change influencing the milk flow rates.

The starting point of milking was taken as the time of application of the fourth teat cup, the recording drum being set in motion at this point. The Ruakura Milk Flow Indicator (Whittlestone, 1954) was used to indicate the beginning of milk flow and the end-point of milking (i.e. when the sightglass became clear, corresponding to a flow of $< 0{\cdot}2$ lb/min). Machine strippings were not considered in these data, so that milking time and associated measures were calculated on a slightly different basis from similar measurements by some previous workers.

For convenience the following terms have been used in this paper.

'Maximum rate', lb/min—maximum milking rate attained in any minute during milking.

'Total time', min—length of time from 'cups on' until the end-point of milking.

'Milking time', min—length of time from the start of milk flow until the end-point of milking.

'Average rate', lb/min—derived figure obtained by dividing the yield by the milking time.

'Time to reach maximum rate', min—length of time from 'cups on' until the start of the maximum milking rate.

RESULTS

The repeatability of the measures of milking rate

A preliminary trial was made in order to assess the accuracy of a single recording of milking rate. Estimates of the repeatability of successive records (measured as the intra-class correlation) for the milking rates of ten cows measured at three consecutive milkings in a single herd, were found to be high for maximum rate of flow (0·73) and milking time (0·86) but somewhat lower for average rate of flow (0·58).

Variation within and between herds for milking rate characteristics

The average within-herd coefficients of variation for the various measures of milking rate are presented in Table 1. The values were very similar, lying between 24·3 and 29·2 %. The number of cows recorded, as a proportion of the total number of cows milked in individual herds, varied considerably (from 8 to 65 %). The herd mean milking rates are therefore estimated to various degrees of accuracy.

The variation amongst mean milking rates for groups of cows in different herds is

also given in Table 1. Analysis of variance showed that 21 % of the variation in maximum rate of flow occurred within herds and the remainder between herds.

Table 1. *Variation within and between herds for milking rate characteristics (measured November–January)*

Measure	Av. coeff. of variation, %	Mean and S.E. of herd averages	Coeff. of variation among herd averages, %
Yield, lb	28·8	10·88 ± 0·65	21·5
Total time, min	24·3	3·45 ± 0·13	14·0
Average rate, lb/min	25·6	3·29 ± 0·15	16·5
Maximum rate, lb/min	29·2	5·38 ± 0·28	18·6

Table 2. *Mean milking rates for groups of herds with similar methods of preparation for milking*

	Good preparation	Average preparation	Poor preparation
Number of herds	2	6	5
Number of cows	40	89	120
Yield of milk, lb	13·72	9·22	11·11
Time to reach maximum rate, min	0·13	0·57	0·70
Total time, min*	2·96	3·27	3·51
Milking time, min*	2·96	3·18	3·43
Average rate, lb/min*	3·63	3·40	3·19
Maximum rate, lb/min*	6·02	5·72	5·67

Group means consist of herd means weighted for number of cows per herd.

* Mean herd values corrected for differences in yield, using the average within-herd regressions on yield.

Good preparation: thorough washing and stripping taken from each teat (30 sec).

Average preparation: short wash and stripping taken from each teat (15–20 sec).

Poor preparation: very brief wash or none.

The influence of preparation for milking on herd milking rates

The average milking rate data are grouped in Table 2 according to the level of stimulus provided before milking. The differences between these groups were confounded by environmental differences between herds. The milking machine vacuum levels for herds were similar (between 15 and 16 inHg) but there were stage-of-lactation effects due to differences in herd sampling dates. For this reason individual milking rates were adjusted for differences associated with differences in yield.

Analysis of covariance of milking rates adjusted for milk yield showed no significant difference between preparation groups ($P > 0{\cdot}1$). There was, however, a significant increase in the within-herd variance for total time and milking time between the good and the average preparation groups, and between the average and the poor preparation groups (Bartlett's Test, Chi-square > 3·84). The increase in total time in some poorly prepared herds was due partly to an increase in the time taken for cows to reach maximum rate of milk flow.

Heritability of maximum rate of flow

The heritability of maximum rate of flow was calculated from the regression of the daughters' records on dams' records on a within-herd basis (thirteen herd groups) and

on a within-sire basis (twenty-eight sire groups for which there was more than a single daughter).

The 150 daughter–dam pairs of records comprised 111 dams with one to five offspring. No full sibs were included in the intra-herd regression (see Kempthorne & Tandon, 1953) and this restriction reduced the number of daughters available by twenty-one.

The heritability estimates on a within-herd basis were calculated by two alternative methods for dealing with data in which dams have more than a single offspring (Table 3). Repeating the dams' record with each daughter's record gave a heritability estimate of 0·87 which was very similar to that obtained by using the mean of a group of daughters' records with their dam's record (heritability 0·84).

Because the animals within herds differed mainly in respect to milk yield (X_1, expressed in pounds) and age (X_2, expressed in lactations) the individual maximum rate recordings (Y) were adjusted for these factors using the multiple regression equation obtained from the data (estimated $Y = 5{\cdot}762 + 0{\cdot}215X_1 - 0{\cdot}006X_2$). However, the heritability estimate obtained using the adjusted data (on a within-sire basis) was approximately the same as that obtained using the unadjusted data (0·70 and 0·77, respectively).

Table 3. *The heritability of maximum rate of flow—daughter on dam regression method*

Method	No. of pairs	Heritability estimate and standard error
Intra-herd regressions		
Repeating the dams' records with each daughter's record	129	$0{\cdot}87 \pm 0{\cdot}14$
Unweighted regression of mean of daughters' records on dams' records	111	$0{\cdot}84 \pm 0{\cdot}16$
Intra-sire regressions		
Repeating the dams' records with each of their daughter's records	110	$0{\cdot}77 \pm 0{\cdot}17$
Using the adjusted data—repeating the dams' records with each daughter's record	110	$0{\cdot}70 \pm 0{\cdot}20$

DISCUSSION

Variation within and between herds

The variations in milking rate characteristics presented in Table 1 are indicative of the variation one would expect to find within and between herds of Jersey cows, and for corresponding measures of milking rate are similar to the results reported by the New Zealand Dairy Board (1950).

The results for the repeatability of the milking rate measures were in general agreement with those of previous workers (Beck, Fryer, & Roark, 1951) who have shown that the characteristic milk removal curve of the individual cow varies little from day to day or even over longer periods. The small number of cows measured in some herds gave rise to large standard errors of herd means. However, the overall average coefficients of variation for the various measures of milking rate were very similar (24–29 %) and it is interesting to note that the highest variation obtained was that for maximum rate of flow.

The differences between mean milking rates for groups of cows in different herds

which comprised approximately 79 % of the total variation in the case of maximum rate of flow, are likely to be partly due to genetic differences and partly to environmental factors such as pulsation rate and ratio, vacuum level and type of udder preparation.

The between-herd variation in maximum rate of flow obtained in this study probably represents a maximum figure, as the within-herd results were from animals more closely related than is usual because of the nature of the samples taken from each herd. Type of preparation and stage of lactation were the only environmental factors measured which varied to any extent among herds.

Technique of preparation

Variation in all milking rate measures is associated with variation in yield (Johansson, 1961) so that the mean milking rates for each group of herds were obtained from the milking rate figures adjusted for differences in yield (Table 2). This adjustment accounted for most of the differences associated with stage of lactation, due to differences in herd sampling dates.

The amount of effort put into preparing the cow appeared to have an influence on all measures of milking rate, although the differences between the means for technique groups were not significant. Poor stimulation methods in some herds resulted in reduced milking rates of some cows but not others, and this affected the variation in milking rate rather than the mean milking rate.

The time spent preparing the cow and the time taken to reach maximum rate of flow were to a certain extent inversely related. In this study a short period of extra preparation was more than made up for by a reduction in the time taken to reach maximum rate of flow and in milking time. This suggests that by improving the stimulation technique for individual cows within a herd worthwhile reductions in milking time could well be obtained by many commercial farmers.

Apart from the effect of yield, maximum rate of flow attained during milking is largely dependent on the size of the teat orifice (Baxter, Clarke, Dodd & Foot, 1950). The maximum rate of flow is thus the best measure when considering the genetic improvement in milking rate, as it appears to be the least affected by the skill and care of the milker.

The heritability estimates

The estimates of heritability obtained in this study from the regression of daughters' records on dams' records were very similar to that (0·74) obtained by Dodd & Foot (1953) from daughter–dam, and sib and half-sib, correlations. Although heritabilities obtained from monozygotic twin data are often over-estimates, those for maximum rate of flow reported by Brumby (1956) and by Donald (1960) are in agreement with the present results.

Kempthorne & Tandon (1953) have suggested the use of a weighting method in cases where dams have more than one daughter. The estimate obtained by this method would be expected to fall between those obtained using the record of the dams repeated with each daughter's record, and the record of each dam with the average of the daughter's records. In this study, as in the study by Bohren, McKean & Yamada (1961), there was only a very small difference between the alternative

methods of estimation, indicating that there would be little to be gained by using the weighting method.

The small differences between the intra-sire and intra-herd estimates of heritability probably result from chance, and the slightly higher intra-sire standard error of estimate was possibly the result of the smaller number of animals available.

Correction for environmental effects is sometimes useful as a means of increasing the efficiency of selection by increasing the heritability (Purser, 1960) but in this study correction of maximum rate of flow records for differences in yield and age made very little difference to the heritability estimate.

The high heritability estimates that were found for maximum rate of flow indicate that this character should respond quickly to selection based on the individual performance of animals.

Considerable variation in milking rate existed within herds, but because this factor is not considered to be of major economic importance, and in view of the small amount of culling and selection that is normally possible, it is probable that the rate of improvement in milking rate is slow. Through the artificial breeding scheme, however, improvement in milking rate could be easily obtained, especially in view of the fact that only a few daughters would be required to constitute a progeny test.

The author wishes to thank Dr D. S. Flux for his helpful advice and criticism. Thanks are also due to Mr J. N. Hodgson and Mr D. Johnston for assistance in contacting the many interested farmers whose co-operation made this study possible.

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Printed in Great Britain

Note on the action of vegetable rennets on sodium caseinate

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(*Received* 17 *October* 1962)

In an earlier paper, one of us (Oosthuizen, 1962) showed that vegetable rennets produce a fall in the viscosity of sodium caseinate solutions which follows the equation of first-order chemical kinetics; but that the proteolysis involved is stronger than that of calf's rennet since the vegetable rennets attack a greater proportion of the casein ensemble.

More recent experiments described in the present note show a further interesting distinction: whereas, with calf's rennet and pure rennin, the proportion of total viscosity $\chi \equiv (\eta_{max} - \eta_{min})/\eta_{max}$ which can be eliminated in the first-order reaction (extrapolating it theoretically to completion) is independent of enzyme concentration (C_e), this is not so for vegetable rennets. Also, contrary to the preliminary findings described by Oosthuizen (1962), with the samples now tested there appears to be no direct proportionality between k_1 and C_e. Plotting double logarithms, the curves relating these variables are approximately linear, but since χ is not independent of C_e, it is not possible to draw any definite conclusions from them about the purity of the enzymes.

The results are shown in Table 1. The concentrations are, of course, only of relative significance and were selected to give the best first-order curves in order to get reliable values of k_1 and χ.

Table 1. *Reaction constants and viscosity changes of sodium caseinate solution acted upon by two vegetable rennets at various concentrations*

A. Ficus *rennet*

C_e	0·15	0·125	0·10	0·05	0·038
$k_1 \times 10^4$	13·8	11·2	9·9	7·2	5·1
χ	0·67	0·63	0·65	0·48	0·14

B. Withania coagulans *rennet*

C_e	0·15	0·11	0·075	0·038
$k_1 \times 10^4$	4·31	3·54	3·25	2·33
χ	0·44	0·43	0·37	0·31

We conclude that, unlike rennin and calf's rennet, these vegetable rennets attack a proportion of the casein which is not only abnormally large but which increases with the increasing concentration of the enzymes.

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Our best thanks are due to Professor K. Iya who sent us the vegetable rennets from India and to Miss Barbara Skidmore (now Mrs Harris) who carried out the viscosity measurements described in this note.

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OOSTHUIZEN, J. C. (1962). *J. Dairy Res.* **29**, 297.

Printed in Great Britain

J. Dairy Res. (1963), **30**, 199

The effect of the replacement of concentrates by roots on the intake and production of dairy cows

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(*Received* 9 *November* 1962)

Summary. To investigate the effect of replacing part of the concentrates by roots, a 16-week winter-feeding experiment was made using twelve Ayrshire cows. All the cows received 8 lb of hay per day and enough grass silage for a measurable residue to be left at each feed. Two rations were supplemented with 45 lb of fodder-beet per day but the other two contained no roots. At each level of root feeding, concentrates were fed at either 3·50 or 1·75 lb per 10 lb of milk.

All the fodder-beet was eaten (8·4 lb dry matter) and, as this caused a reduction in the intake of silage dry matter of only 3·4 lb per day, there was a net increase in dry-matter intake. The level of concentrate feeding had little effect on the intake of the other feeds. Rations which included roots contained significantly less crude fibre than rations without roots.

The mean daily milk yields for the treatments with the low and high concentrate levels were 29·5 and 33·9 lb, respectively, without roots and 31·8 and 35·8 lb with roots. The fat percentages of the milk were similar on all four treatments whereas the solids-not-fat (s.n.f.) increased significantly from 8·42 % on the treatment without roots and low concentrates to 8·64 % on the treatment with roots and high concentrates. The response per 1 lb of extra s.e. was 1·0 lb milk and 0·02 % s.n.f. between the low and high concentrate treatments and 0·7 lb milk and 0·05 % s.n.f. between the two root treatments. The analysis of milk from individual quarters of all the cows during each period showed the continuous presence of some subclinical mastitis. It was estimated that this lowered the general level of s.n.f. percentage by 0·2 % but did not bias the results from any one feeding treatment. For milk from healthy quarters only, the response to 1 lb extra s.e. from concentrates was 0·03 % s.n.f. and from beet it was 0·08 % s.n.f.

During the winter of 1959–60 a cattle feeding trial was conducted at this Institute to investigate the effect of adding roots to a diet consisting of hay, grass silage and concentrates (Castle, Drysdale & Waite, 1961). The results showed that the addition of roots increased the total daily intake of dry matter and on average for each 1 lb of root dry matter eaten, there was a reduction of only 0·45 lb of dry matter in the amount of other feeds consumed, mainly silage. Because of this increase in dry-matter consumption, increases in milk yield and in the s.n.f. percentage of the milk were found also. In other feeding trials at this Institute involving rations without roots it has been shown that roughages such as hay and grass silage could often

impose a physical limit on the dry matter intake of the cattle (Holmes, Reid, MacLusky, Waite & Watson, 1957; Castle & Watson, 1961). It was thus virtually impossible for a reduction in the amount of concentrate dry matter to be compensated for by an increase in roughage dry matter. It was thought possible, however, that a reduced intake of concentrates could be fully replaced, at least on a dry-matter basis, if roots were included in a ration containing hay and silage.

To investigate this possibility a short-term feeding trial was made in which a basal diet of hay and grass silage was fed either with or without roots and in addition concentrates were rationed at either a high or a low level. The first object of the trial, which is described below, was to measure the differences in the feed intake of the cows, and the second to see what effects these changes would have upon milk yield and milk quality.

EXPERIMENTAL

Experimental design

Twelve Ayrshire cows in the Institute herd were used. Five cows were in their second to fourth lactation and the other seven in their fifth to ninth lactation, which gave an average of 4·5 lactations per cow. For a minimum period of 4 weeks before the experiment started the cows were on a diet of hay, silage and concentrates and during the week just before the experiment the animals were fed silage, hay, fodder-beet and concentrates similar to those given during the experiment. The cows were housed throughout the experiment in a conventional byre with facilities for individual feeding, but were allowed into a bare concrete yard each day from 10.00 a.m. to 12.00 noon.

The twelve cows were divided into three groups of four so that the animals in each group were at a similar stage of lactation. The four cows were allotted at random to one of four feeding treatments in a 4×4 balanced Latin square layout and the experimental periods were of 4 weeks' duration. In an attempt to eliminate the effect of stage of lactation on milk quality the starting date for each group was staggered and was on average 64 days after calving. This allowed each group to complete the experiment between the second and sixth month of lactation as suggested by Bailey (1952) in order to overcome any effect of pregnancy on the S.N.F. content of the milk. The experiment started on 13 November 1961 and ended on 8 April 1962. The change from one treatment to another was made over 4 days and in calculating the results of the trial the data from weeks 3 and 4 in each period were used.

Feeding

The cows on all the treatments were offered grass silage at 7.00 a.m. and 12.00 noon and 8 lb of hay at approximately 5.00 p.m. each day. A sufficient quantity of silage was offered to ensure that a residue was always available for weighing 3 h after feeding. In addition to the hay and silage the cows received the following four treatments.

Treatment	Fodder-beet, lb per cow per day	Concentrates, lb/10 lb milk
A	45	3·50
B	45	1·75
C	Nil	3·50
D	Nil	1·75

The fodder-beet on treatments A and B was given in three feeds of 15 lb each and was fed at the same time as the silage and the hay, i.e. at 7.00 a.m., 12.00 noon, and 5.00 p.m.

The weight of concentrates fed in period 1 was calculated from the mean daily yield of milk in the week before the experiment began. In periods 2, 3 and 4 the amount fed was based on the equalized feeding principle of Lucas (1943) using the average daily milk yield from the last week of the previous period and the average decline in milk yield of the group. The amount of concentrates given in any one period was kept constant throughout the 4 weeks. Half the weight of concentrates was given at each milking time. The concentrate mixture consisted, in parts by weight, of ground barley $4\frac{1}{2}$, bruised oats 2, groundnut cake 2, flaked maize $1\frac{1}{2}$, kibbled locust beans $\frac{1}{2}$ and mineral pre-mix $\frac{1}{4}$.

Table 1. *The composition and calculated nutritive value of the feeds*

	Dry matter, %	Percentage of the dry matter						
		Crude protein	Ether extract	Nitrogen-free extract	Crude fibre	Ash	Starch equivalent	Digestible crude protein
Concentrates	85·0	21·6	3·7	64·2	6·0	4·5	79	17·8
Hay	84·6	7·7	2·2	54·5	30·6	5·0	51	4·6
Grass silage	19·5	12·0	4·2	42·6	32·5	8·7	50	7·3
Fodder-beet	18·8	7·9	0·6	80·7	5·1	5·7	54	3·6

The hay was made from a timothy and meadow-fescue sward cut on 12 June. After partial drying in the field the hay was baled and dried finally on a grass drier converted for barn-hay drying, the whole process taking 4 days. The resultant hay was green and leafy and in excellent condition without any weathering or damage in the field. The silage was made in late May from grass cut at the long leafy stage. The variety of fodder-beet was Red Øtofte XI. The analyses and feeding values of these feeds and of the concentrate mixture are given in Table 1.

All the feeds were weighed individually every day throughout the trial, and on 5 consecutive days each week the quantities of each feed refusal were weighed also. The feeds were sampled as in the earlier trial (Castle *et al.* 1961).

Digestibility trials using the total collection method were made on the four feeds with four Ayrshire bullocks. These were on a planned feeding regimen with faeces collected over periods of 10 days, and four determinations were made for each feed. Digestibility coefficients were calculated directly for the hay and silage, and by difference for the concentrates and fodder-beet; S.E. values were calculated for each feed from the chemical analyses and digestibility data. The S.E. value for the hay was calculated using a deduction of 0·58 units of S.E. per 1 % of crude fibre, but the final S.E. value was not increased by 20 % as in Woodman's tables (Evans, 1960) because the hay was an excellent product. To calculate the S.E. of the fodder-beet a factor of 0·83 was used to convert the content of digestible N.F.E. into a weight of S.E. as the fodder-beet dry matter contained 70 % soluble sugars, mainly sucrose. It is recognized that the calculation of the S.E. and D.C.P. contents of the feeds is somewhat arbitrary, but it is useful to compare the intakes on the experimental

rations with the requirements of the animals as estimated by the feeding standards calculated in the same way (Evans, 1960).

Milk yield, milk quality and live weight

The cows were milked twice a day and their yields recorded to the nearest $\frac{1}{4}$ lb. Samples of milk from consecutive evening and morning milkings were taken twice a week (Monday and Tuesday, and Thursday and Friday) from each cow in weeks 3 and 4 of each period. The evening and morning samples were mixed in proportion to yield and analysed for fat and total solids. In order to isolate the true effect of the feeding treatments free from the effects of udder disease (Waite, 1961), individual quarter milking of each cow was done at the afternoon milking on Tuesday and Friday in the last week of each period to provide samples for chemical analyses and determination of the total and differential cell count (Blackburn, Laing & Malcolm, 1955). Also fore-milk from each quarter was examined fortnightly for the presence of pathogenic bacteria.

The cows were weighed at approximately 11.45 a.m. on Wednesday, Thursday and Friday in week 4 of each period.

RESULTS

Feed consumption

Dry matter

The mean weight of dry-matter intake contributed by the various feeds on the four treatments and the total daily intake of dry matter are given in Table 2. As expected, there were wide variations, ranging from 24·3 lb/day on treatment D to 34·2 lb/day on treatment A.

The fodder-beet was readily consumed by the cows (treatments A and B) and the total daily intake of dry matter was significantly higher ($P < 0{\cdot}01$) than on rations without it (treatments C and D). The increase in dry-matter consumption of 5 lb/day as a result of the inclusion of 8·4 lb fodder-beet dry matter at both the low and the high levels of concentrate feeding resulted from a decrease in the amount of silage eaten, the intake of hay and concentrates being almost unaffected. The daily consumption of silage dry matter fell significantly ($P < 0{\cdot}01$) from 11·5 lb on treatment C to 8·1 lb on treatment A and from 12·2 lb on treatment D to 8·6 lb on treatment B.

The high level of concentrate feeding raised the total dry-matter intake of the animals by 4·9 lb/day whether beet was included or not. There was a small non-significant decrease in the intake of silage dry matter as a consequence of the increased concentrate intake but the intake of hay was virtually unaffected.

As both the raising of the level of concentrate feeding and the inclusion of fodder-beet in the ration had almost identical effects on the dry-matter intake of the cattle, the intakes on treatment B (low concentrates plus fodder-beet) and treatment C (high concentrates and no fodder-beet) were almost the same.

The intake of dry matter per cow per day expressed as a percentage of mean live weight is also given in Table 2. This value was increased significantly ($P < 0{\cdot}01$) by an average of 0·5 % by the inclusion of fodder-beet in the diet and by 0·4 % by increasing the level of concentrate feeding, the increases being cumulative.

Table 2. *The feed and nutrient intakes on the four feeding treatments*

Treatment	Dry matter, lb per cow per day					Daily dry matter consumption as % of live weight	Starch equivalent		Digestible crude protein		Crude fibre, lb/day
	Fodder-beet	Concentrates	Hay	Silage	Total		lb/day	% of Woodman's standard *	lb/day	% of Woodman's standard*	
A	8·2	11·4	6·5	8·1	34·2	3·2	20·8	131	3·2	106	5·7
B	8·5	5·6	6·6	8·6	29·3	2·8	16·6	114	2·2	88	5·6
C	—	11·0	6·7	11·5	29·2	2·7	17·9	117	3·1	108	6·5
D	—	5·4	6·7	12·2	24·3	2·3	13·8	98	2·1	82	6·4
Standard error of a difference between two means	—	±0·33	±0·09	±0·41	±0·46	±0·03	±0·29	±2·0	±0·06	±4·2	±0·12

* Evans (1960).

Starch equivalent and digestible crude protein

The average daily intakes of S.E. and D.C.P. by weight and as a percentage of the cows' requirements according to the standards of Woodman (Evans, 1960) are presented in Table 2. These were calculated from the mean intake of each feed and from the values given in Table 1. The intake of S.E. was increased significantly ($P < 0{\cdot}01$) either by feeding fodder-beet or by increasing the level of concentrates. On treatment C (high concentrates and no fodder-beet) the intake of S.E. was higher than on treatment B (low concentrates plus fodder-beet) although the values were not statistically different when expressed as a percentage of the Woodman requirements.

The inclusion of fodder-beet in the ration did not significantly increase the weight of D.C.P. in the ration, whereas the higher level of concentrates increased the amount on average from 85 to 107 % of the Woodman standard, statistically a highly significant effect.

Crude fibre

The mean daily intake of crude fibre (Table 2) was calculated from the daily dry-matter intakes and the values given in Table 1. Changes in the level of concentrate feeding had no significant effect on the intake of crude fibre but on both treatments A and B, which included fodder-beet, the cows consumed significantly less crude fibre than on treatments C and D.

Animal production

Animal health

The general health of the cows during the trial was excellent and digestive disorders which are occasionally reported with fodder-beet feeding were completely absent. Four cows had persistent staphylococcal infections in three quarters throughout the experiment and failed to respond to antibiotic treatment. Similarly, another cow had persistent streptococci in the milk of one quarter. Judged by cell counts and bacteriological examination four other cows had subclinical mastitis at various times. In all cows the usual signs of clinical mastitis, heat, pain or swelling of the udder or clots in the milk were absent throughout. The effect of udder health on milk composition is briefly discussed later in this paper and more fully in the following communication (Waite, Abbot & Blackburn, 1963).

Milk yield

The average daily milk yields per cow on each treatment are given in Table 3. Throughout the trial, yields were at a satisfactory level and substantial differences were recorded between treatments. On average the yield of milk was significantly higher ($P < 0{\cdot}01$) by 4·2 lb/day when the level of concentrate feeding was increased from the low to the high level. The increase in yield as a result of the fodder-beet feeding averaged 2·1 lb/day and was also significant. These increases in the yield of milk were additive. Treatment B, containing fodder-beet and a low level of concentrates, gave an average yield of 31·8 lb/day, which was significantly lower than the yield on treatment C, 33·9 lb/day, which supplied no fodder-beet but a high level of concentrates.

Milk composition

(*a*) Whole udder samples.

The average fat and S.N.F. percentages of the milk for the four treatments are given in Table 3. The fat percentage of the milk on all the treatments was high and there were no significant differences between treatments. The S.N.F. values showed progressive increases from 8·42 % on treatment D to 8·64 % on treatment A. The largest differences were between rations with and without fodder-beet, and averaged 0·14 %, which was statistically significant ($P < 0·01$). The differences in the S.N.F. content caused by the two levels of concentrate feeding averaged 0·08 %, which was not quite significant ($P > 0·05$).

Table 3. *The mean daily milk yield, chemical composition of the mixed milk from the whole udder and the mean live weight of the cows*

Treatment	Milk yield, lb per cow per day	Milk composition, %		Live weight, lb
		Fat	S.N.F.	
A	35·8	4·30	8·64	1087
B	31·8	4·29	8·56	1056
C	33·9	4·23	8·50	1082
D	29·5	4·20	8·42	1052
Standard error of a difference between two means	±0·60	±0·07	±0·04	±7

(*b*) Quarter samples.

The presence of bacterial infection in a substantial proportion of quarters lowered the lactose percentage (and hence the S.N.F. percentage) and the casein number of the milk from those quarters. Because the worst affected cows were in this condition throughout the whole of the experiment the deleterious effect on milk composition was fairly evenly spread over all feeding treatments and no unequal bias was introduced. For milk from only those quarters judged to be healthy the S.N.F. values for the four feeding treatments were treatment A, 8·87 %, B, 8·75 %, C, 8·68 % and D, 8·57 %, each value being about 0·2 % higher than those shown in Table 3.

Live weight

The average live weights of the cows on the four treatments are also given in Table 3. The fodder-beet had no significant effect on live weight, whereas the higher level of concentrates irrespective of root feeding significantly increased live weights ($P < 0·01$).

DISCUSSION

The results of this experiment agree well with those found in the earlier trial at this Institute when fodder-beet with a high dry-matter content was added to a basal diet of hay and silage (Castle *et al.* 1961). In the present trial the addition of 45 lb of fodder-beet (8·2–8·5 lb of beet dry matter) to the daily ration increased the total dry-matter intake by 5·0 lb, and this occurred at both a low and a high level of concentrate feeding. As expected, the fodder-beet was consumed readily by the

cattle but they did not refuse an equal and compensating weight of dry matter from the other feeds. The daily consumption of hay was virtually the same on rations either with or without fodder-beet and, as in the previous trial, the largest effect was on the silage part of the ration which decreased by 30 % at both levels of concentrate feeding when beet was offered. On average, for each 1 lb of beet dry matter eaten, there was a reduction of 0·40 lb of dry matter in the amount of basal feed consumed, which is a value similar to that of 0·45 lb found in the previous experiment (Castle *et al.* 1961).

The average weight of dry matter eaten per day, expressed as a percentage of live weight, was 3·0 % on the two treatments that included fodder-beet and only 2·5 % on the treatments without beet. The beet was an extremely palatable feed and was always selected and consumed before either the hay or the silage when both feeds were offered together in the trough. The total weight of crude fibre in the rations without roots (treatments C and D) was 6·4 lb/day, which gives an average crude fibre percentage for the entire ration of 24, whereas on the treatments with roots (treatments A and B) the crude fibre intake was 5·6 lb and the percentage of crude fibre for the entire ration was 18. This value is close to the optimum of about 16 % which Russell (1953) suggests should not be exceeded for high yielding cows.

The increases in the consumption of S.E. from treatment D to treatment A (Table 2) were accompanied by increases in the milk yield (Table 3). The overall response per 1 lb of extra S.E. was 0·92 lb milk, but this average value obscured a difference between the effect of S.E. derived from concentrates and that from fodder-beet. When the level of concentrate feeding was increased from 1·75 to 3·50 lb per 10 lb of milk (treatments B + D compared with A + C) both the S.E. and the D.C.P. intakes were increased and the response per 1 lb of additional S.E. was on average 1·0 lb of milk. When the fodder-beet was added to the diet (treatments C + D compared with A + B) the S.E. intake was increased but the D.C.P. intake was virtually unchanged and the response per 1 lb of extra S.E. averaged only 0·74 lb of milk. The effect of the extra S.E. in increasing the S.N.F. content of the milk was evident also in this trial with a greater response from roots than from concentrates. For 1 lb of additional S.E. from concentrates the response was 0·02–0·03 % S.N.F., whereas when beet was added to the ration the response was 0·05–0·08 %. The latter value of 0·05 % S.N.F. is the same as that recorded in the previous trial (Castle *et al.* 1961) when the additional S.E. was all derived from fodder-beet. The present experiment adds weight therefore to the suggestion then made that roots, with their high sugar content, are an extremely useful supplement to diets containing silage, particularly where the S.N.F. percentage of the milk is low.

The object of analysing the milk of each cow by quarters was to guard against an accidental biasing of the results attributed to a feeding treatment by variations in milk composition caused by subclinical mastitis. The combined evidence of cell count, chemical composition and bacteriological examination showed that a high proportion of quarters was affected, most of them for the whole period of the experiment. The effect of the disease therefore was to lower the level of milk composition in general, rather than to influence the result of an individual feeding treatment.

One of the original aims of the experiment was to investigate the extent to which fodder-beet could compensate for a low level of concentrate feeding when it is known

that roughages such as hay and silage cannot fully make up a deficit of concentrates in the ration. This involves a comparison of treatments C and B (Table 2) and it will be seen that the inclusion of 45 lb of fodder-beet/day in the ration fully made up the deficiency of concentrate dry matter. On treatment C, without roots but with the high level of concentrates, the intake was 29·2 lb of dry matter/day, and on treatment B, with roots and a low level of concentrates, it was 29·3 lb/day. As a result of the lower S.E. and D.C.P. of the fodder-beet dry matter compared with the concentrate dry matter, the intake of nutrients on treatment B was lower than on treatment C, particularly in D.C.P., 2·2 lb/day on treatment B compared with 3·1 lb on treatment C. Because of these differences the mean daily milk yield on treatment B, 31·8 lb/day, was significantly lower than on treatment C, 33·9 lb/day, although both the fat and the S.N.F. percentages were higher on treatment B than on treatment C. The fodder-beet therefore, although compensating on a dry-matter basis for the 50 % reduction in the use of concentrates, did not produce an equal milk yield. The difference between the value of the milk and the cost of the daily ration at current prices for feed showed that treatment B was slightly more profitable than treatment C. Such calculations are dependent on fluctuating prices charged for each feed, but it is clear that if the cost of the concentrates increased and the price for the milk either remained steady or fell slightly there would be an increasing advantage for treatment B compared with treatment C, especially where quality milk payments are made.

The inclusion of fodder-beet in the ration serves two purposes; it increases the total dry-matter intake whilst at the same time reducing the amount of fibre and it also provides more readily available energy which improves milk yield and the S.N.F. percentage. The increased dry-matter intake could be achieved by making a silage with a higher dry-matter content but, as recent work shows (Brown, 1962), there might be no improvement in milk yield and little or none in milk composition, probably because the pattern of the rumen fermentation would remain the same. Thus, even with high dry-matter silage, the addition of roots to the ration would still increase dry-matter intake, and in all likelihood the yield and S.N.F. percentage of the milk.

The authors thank Dr P. S. Blackburn for making the cell counts and bacteriological examinations of the milk, and Mr N. H. Strachan and Mr J. Abbot for the chemical analyses of the milk and feeds. They gratefully acknowledge also the help of the members of the Dairy Husbandry Department of the Institute who did much practical work and computing.

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Printed in Great Britain

The use of quarter samples in the assessment of the effects of feeding treatments on milk composition

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(*Received* 17 *January* 1963)

Summary. To investigate whether milk of abnormal quality from some quarters of twelve cows on a Latin square type feeding trial would bias the results for chemical composition, the compositions of milk from normal and affected quarters were compared with each other and with that of the milk obtained from the whole udder by normal milking.

The average number of quarters producing abnormal milk in each of the four periods was eighteen, with only small variation between periods. Cell counts ranged from 50000 to 25×10^6/ml with 31 % of them below 500000/ml; as cell count increased, lactose percentage and casein number fell. Pathogenic bacteria were detected in only 70 % of the abnormal milks. The solids-not-fat (S.N.F.) percentage of milk from affected quarters was lower on average than that from healthy quarters by 0·43, the decrease resulting entirely from a lowered lactose percentage. The total nitrogen content was the same in both types of milk, but there was 4·5 % less casein in milks from affected quarters.

Because in this particular experiment the distribution of the abnormal milks was almost uniform over the four feeding treatments, the results obtained for treatment effect on milk composition by the normal milking method were not biased by the occurrence of disease, but the general level of composition was depressed by 0·2% fat and 0·2 % S.N.F. In other experiments the incidence of disease might happen by chance to differ between treatments, with the result that the findings about milk composition could be erroneous.

In a previous note (Waite, 1961) the possibility was discussed that the results for milk composition from feeding trials with small numbers of cows might be inaccurate because of the effect of undetected udder damage or mastitis of the so-called subclinical type. Examples were given of affected quarters which produced milk having a lactose percentage only 70–80 % of that of the milk from the healthy quarters of the same udder at the same milking, and in which the casein number had fallen by 16–18 units. In a more detailed study of the milk from a cow, which *post mortem* was found to have had an undetected abscess in each of two quarters (Waite & Blackburn, 1963), the percentages of lactose and casein in milk from these quarters were seriously lowered and the level of blood serum proteins in the milk raised considerably.

Cows such as those detailed in these communications show none of the signs of clinical mastitis such as heat, pain or swelling of the quarter and the milk does not contain clots. Pathogenic bacteria are frequently present in the milk at some time and the cell count is usually abnormally high, consisting of a large proportion of polymorph (pus) cells. The disease, in short, is in a mild form which may or may not develop further and at this stage will be referred to as subclinical mastitis. The poor chemical quality of the milk of such cows may often therefore be erroneously dismissed as the result of inadequate feeding or of an inferior genetic strain. In experimental work, however, chance infections or mechanical damage to the udder could invalidate the results for the effect of feeding treatment on milk composition (and possibly also on milk yield) if more cows were affected when on one treatment than on another. During the course of a recent feeding trial the opportunity was therefore taken to investigate this problem further.

EXPERIMENTAL

The design of the trial and a description of the cows, their feeding and management have been given in the preceding paper (Castle, Drysdale, Waite & Watson, 1963). The milks for the present study were taken with a bucket quarter milking machine from all the cows on two evenings in the final week of each treatment period and were analysed for total solids, fat, lactose, and total and casein nitrogen by methods previously described (Waite, White & Robertson, 1956). The total and differential cell counts of these samples were determined (Blackburn, Laing & Malcolm, 1955) and fore-milk from each quarter examined fortnightly for the presence of bacteria by the method of Blackburn (1956).

The criteria used to decide whether a milk from any quarter was abnormal were substantially the same as those suggested previously (Waite, 1961), i.e. (*a*) a total cell count of more than 100000/ml in the milk of the fully milked quarter, (*b*) a lactose percentage at least 0·15–0·20 units lower, and (*c*) a casein number at least 2–3 units lower than in the milk of the highest chemical composition from any other quarter of the udder at the same milking, and (*d*) the presence of staphylococci, streptococci or coliform organisms in the fore-milk.

RESULTS

In the month preceding the experiment it was found that eight of the forty-eight quarters, one from each of five cows and three from another, were producing milk of abnormal composition. During the 16 weeks of the experiment more quarters became affected, giving totals for the twelve cows of fifteen in Period 1, seventeen in Period 2, nineteen in Period 3 and twenty-one in Period 4. None of these cows exhibited the signs of clinical mastitis. The quarters producing abnormal milk are detailed in Table 1 from which it can be seen that four cows, nos. 9–12, contributed half or more of the abnormal samples in each period.

Not all the samples classed as abnormal exhibited all four criteria of abnormality (Table 1). A lowered lactose percentage and casein number occurred in all the abnormal samples and a high cell count in 85% of them, but pathogenic bacteria were present in only fifty-six of the eighty abnormal samples (70%). Antibiotic

treatment was given when coagulase positive staphylococci or any streptococci were detected, and although this frequently was without any marked effect it did in some cows temporarily remove bacteria from subsequent milk samples and reduce the cell count. Antibiotic treatment brought no improvement in the chemical composition of the milk.

Table 1. *The number of quarters producing milk of abnormal composition in each period*

Cow no.	Period				
	Preliminary	1	2	3	4
1	0	0	0	0	0
2	0	0	0	1	1
3	0	0	1	1	0
4	0	0	1	1	1
5	0	1	1	1	1
6	0	1	1	1	2
7	1	1	1	1	1
8	1	1	1	3	3
9	1	2	3	2	3
10	1	3	2	2	3
11	1	3	3	3	3
12	3	3	3	3	3
Total	8	15	17	19	21 = 80
Four criteria fulfilled	5	8	11	12	13 = 49
Three criteria only fulfilled	3	4	3	5	7 = 22
Two criteria only fulfilled	0	3	3	2	1 = 9

The cell counts have been arbitrarily grouped in five ranges in Table 2 and are shown for each period of the experiment. It can be seen that although abnormal in chemical quality, eleven (14 %) of the samples contained less than 100000 cells/ml. Most of these occurred in the milk sample which first gave indications of abnormality.

The amount by which the lactose content in these samples fell below that in the milk of the unaffected quarters of the same udder at the same milking was recorded, and the average for each cell count group is included in Table 2. There was considerable variation about the averages, but it is clear that even cell counts below 100000/ml in these abnormal samples were associated with an appreciable decrease in lactose percentage, and that above 100000/ml the lactose percentages were seriously lowered. All four types of bacteria listed in the lower part of Table 2 occurred in the fore-milk of quarters which gave milk of abnormal chemical quality. Neither coagulase positive staphylococci nor any streptococci were present in milks in which the cell count was below 100000/ml.

A comparison has been made between the average chemical composition of the milk from the healthy and the affected quarters of each cow taken at the same milkings during the 16 weeks of the experiment. These results are given in Table 3, where the number of quarters producing the milks is also shown. (Cow no. 1 had no affected quarters in any period.) The biggest differences were in the milks from cows

with two or three affected quarters, leading to a decrease in the S.N.F. percentage of approximately 0·6. These lower S.N.F. values were brought about entirely by a corresponding decrease in the lactose percentages, the crude protein values (total N $\times$ 6·38) being essentially the same in both sets of milks. The proportion of the total nitrogen contributed by casein in the milk from affected quarters (casein number) was about 10 % less than that in the milk from healthy quarters. The fat percentage in the milk from the affected quarters was also lowered appreciably.

Table 2. *The distribution of cell counts, the decrease in lactose percentage and the incidence of bacteria in milk from affected quarters*

		Cell count/ml milk				
Period	No. of affected quarters	Below 100 000	100 000 to 500 000	500 000 to 1 000 000	$1–5 \times 10^6$	$> 5 \times 10^6$
Preliminary	8	1	2	2	2	1
1	15	3	4	2	3	3
2	17	4	6	3	4	—
3	19	2	9	1	6	1
4	21	1	9	2	7	2
Total	80	11	30	10	22	7
	Average decrease in lactose percentage in each cell count group					
Preliminary	8	0·10	0·49	0·51	0·72	1·00
1	15	0·25	0·23	0·56	0·61	0·89
2	17	0·22	0·42	0·35	0·69	—
3	19	0·25	0·33	0·76	0·60	1·03
4	21	0·31	0·54	0·84	0·77	0·61
	Average*	0·23	0·41	0·56	0·62	0·84
	The incidence of bacteria† in each cell count group					
Preliminary	3	0	1S	0	1 Str	1S
1	11	1m	3m, 1S	2m	3S	1 Str
2	12	2m	2m, 3S	1m, 1S	3S	—
3	15	2m	3m, 1S	1 Str	6S	1S, 1 Str
4	15	1m	1m, 2S, 1C	1S	4S, 3 Str	2S
Total	56	6	18	6	20	6

* Weighted for the number of samples.

† m, coagulase negative staphylococci; S, coagulase positive staphylococci; Str, streptococci; C, coliform organisms. The number preceding the letter refers to the number of samples so infected.

The relationship between lactose percentage (which would be only slightly affected by feeding treatment) and log cell count was examined statistically for each cow, using the results from quarters giving both normal and abnormal milk. The regression coefficient, the standard error of the coefficient and the degree of statistical significance as indicated by the analysis of variance are given in Table 4. As would be expected, where a cow produced little or no abnormal milk during the experiment there was no significant relationship and the regression coefficient was small, but in the badly affected cows (nos. 9–12) a tenfold increase in cell count led to a fall in the lactose percentage (and hence in S.N.F. percentage) of from 0·1 to 0·5 %.

In Table 5 the analyses of the normal and abnormal milks from the eleven cows with the quarters giving abnormal milk have been grouped by feeding treatment (for

details see preceding paper by Castle *et al.* 1963). There were seventy-two abnormal and 104 normal samples, the abnormal milks occurring eighteen, nineteen, eighteen and seventeen times in treatments A to D, respectively. Because of the disproportionately large contribution of abnormal milks made by four cows throughout the whole experiment (nos. 9–12 in Tables 1 and 2), the lowered S.N.F. and lactose per-

Table 3. *A comparison of the chemical composition of milk from the healthy quarters with that of the milk from the affected quarters of the same cows*

Cow no.	Quarters	No. of quarters	Fat, %	S.N.F., %	Lactose, %	Crude protein, %	Casein no.
2	Healthy	3	5·66	8·79	4·55	3·28	79·1
	Affected	1	5·48	8·58	4·28	3·38	76·7
3	Healthy	3	4·20	8·75	4·68	3·18	79·3
	Affected	1	4·09	8·68	4·60	3·16	77·6
4	Healthy	3	4·98	8·90	4·88	3·12	80·8
	Affected	1	4·78	8·57	4·59	3·09	78·5
5	Healthy	3	5·68	9·11	4·46	3·73	79·5
	Affected	1	5·67	8·74	4·19	3·75	76·8
6	Healthy	3	4·81	9·01	4·48	3·66	78·4
	Affected	1	4·53	8·65	4·13	3·64	75·5
7	Healthy	3	5·52	8·65	4·45	3·28	76·0
	Affected	1	5·05	8·31	4·09	3·29	70·9
8	Healthy	2	5·41	8·54	4·40	3·20	75·2
	Affected	2	4·95	7·98	3·77	3·26	69·2
9	Healthy	2	4·58	8·67	4·61	3·12	77·6
	Affected	2	4·24	8·08	4·03	3·09	71·5
10	Healthy	1	4·69	8·47	4·78	2·79	77·9
	Affected	3	3·95	7·83	4·27	2·71	70·7
11	Healthy	1	4·16	8·91	4·68	3·32	79·1
	Affected	3	3·79	8·43	4·21	3·33	73·7
12	Healthy	1	4·42	8·53	4·65	3·01	74·7
	Affected	3	3·66	7·74	3·86	2·98	66·9
	Mean—Healthy		4·92	8·76	4·60	3·24	78·0
	Mean—Affected		4·56	8·33	4·18	3·24	73·5

Table 4. *The regression of lactose percentage (y) on log cell count (x)*

Cow no.	Regression coefficient	S.E. of coefficient	Significance of F value
1	−0·005	±0·010	N.S.
2	−0·066	±0·030	*
3	−0·070	±0·071	N.S.
4	−0·004	±0·018	N.S.
5	−0·285	±0·045	*
6	−0·051	±0·045	N.S.
7	−0·463	±0·047	**
8	−0·025	±0·021	N.S.
9	−0·391	±0·051	***
10	−0·545	±0·107	***
11	−0·133	±0·027	***
12	−0·354	±0·067	***

N.S. = not significant.
* = $P < 0·05$; ** = $P < 0·01$; *** = $P < 0·001$.

centages and casein numbers were distributed fairly equally between the four feeding treatments. Feeding treatment effects therefore followed the same pattern in the milk from both affected and healthy quarters, although in the abnormal milks the compositional level was lower by 0·37–0·51 % S.N.F., 0·43–0·60 % lactose, 3·6–7·4 casein number units and 0·35–0·47 % fat. In treatment D the effect was slightly greater than in the other three treatments. When the composition of the milk from the healthy quarters of all the twelve cows was compared with the results of Castle *et al.* (1963) for the milk of all the quarters, the former contained approximately 0·2 % more S.N.F.:

Treatment	A	B	C	D
Healthy quarters (12 cows)	8·87	8·75	8·68	8·57
Mixed milk (Castle *et al.* 1963)	8·64	8·56	8·50	8·42

Allowing for the higher fat percentage in the present (evening) milks it is probable that the presence of the abnormal milks depressed the fat percentages recorded by Castle *et al.* by about half the difference between those for normal and abnormal milk (Table 4), i.e. by about 0·2 %.

Milk yield. When the milk yields of the affected quarters were inspected it was clear that an appreciable loss of milk had occurred, but it was difficult to be certain of the amount because of the possibility that compensation had occurred in the corresponding healthy quarter. Since all except one cow (no. 12) started the lactation free from disease the probable loss in milk yield has been estimated by comparison with the proportionate yield of normal quarters. For the eleven cows with affected quarters the average loss in milk was about 14 % of their recorded yield, or 4–5 lb per cow per day, assuming no compensation to have taken place.

Table 5. *The effect of feeding treatment on the composition of milk from healthy and affected quarters*

Treatment	Quarters	Fat, %	S.N.F., %	Lactose, %	Crude protein, %	Casein, %	Casein no.
A	Healthy	4·97	8·88	4·64	3·34	2·61	78·1
	Affected	4·64	8·44	4·18	3·33	2·48	74·5
B	Healthy	4·94	8·76	4·64	3·22	2·51	78·8
	Affected	4·50	8·34	4·17	3·24	2·36	73·7
C	Healthy	4·92	8·66	4·63	3·18	2·47	77·7
	Affected	4·45	8·29	4·21	3·22	2·36	73·3
D	Healthy	4·97	8·60	4·60	3·11	2·42	78·1
	Affected	4·60	8·09	4·00	3·18	2·25	70·7

DISCUSSION

The considerable amount of milk of abnormal composition produced by some quarters in the feeding trial described by Castle *et al.* (1963) did not affect the estimate of the effect of the four rations on the chemical composition of the milk. This was because on each feeding treatment the numbers of abnormal samples were very similar and the four cows worst affected were in that condition throughout the whole experiment. This seems an additional advantage of the Latin square design of such feeding trials and a very different result could have been obtained in a trial where all cows did not receive all the feeding treatments.

The evidence provided by the cell counts and bacteriological examination indicates that most of the abnormality in chemical composition was the result of subclinical mastitis. Although the effect of this condition is usually to lower the S.N.F. percentage of the milk, here there was also a marked decrease in fat percentage. However, if the poorer composition of such abnormal milks results from an infiltration of blood serum it could be expected to dilute both fractions.

The results emphasize the part that udder damage or infection can play in lowering the chemical quality of the milk not only of individual cows but also of a group of twelve, which is the size of many small herds.

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Printed in Great Britain

A note on the effect of heat on the colour of goat's milk

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(*Received* 20 *November* 1962)

Summary. Goat's milk heated at temperatures in the range 94–120 °C underwent the same colour changes as previously found for cow's milk, i.e. whitening followed by browning. The rate of browning varied with temperature according to Arrhenius's Law, and had a Q_{10} between 95 and 120 °C of 2·6–2·7. This is slightly lower than the value found for cow's milk. It was difficult to produce whitening curves for milk heated at 75–100 °C because of unexplained reflectance variations. It appeared that the amount of whitening which could be produced became less as the interval after milking increased.

The changes in the colour of cow's milk which occur on heating have been studied in some detail (Burton, 1954, 1955*a*, *b*). Some general results have now been obtained on the corresponding changes in goat's milk.

EXPERIMENTAL

Samples of milk (each of 10 ml) were heated in thin-walled aluminium tubes, 9·5 cm long and 1·5 cm diameter: rubber inserts in the screwed caps of the tubes prevented leakage at treatment temperatures above 100 °C. The tubes were immersed for heating in a thermostatically controlled, electrically heated oil bath. The time required for the milk to reach the bath temperature (approximately 2 min) was neglected in stating the treatment time. The tubes were cooled in flowing tap water for 2 min and finally placed in water at room temperature for at least 1 h before colour measurement, to allow the reflectance of the milk to reach equilibrium at the value corresponding to room temperature (Burton, 1956*a*).

The colour of the milk was determined as its spectral variation of reflectance, using an EEL reflectance spectrophotometer (Evans Electroselenium Limited). The reflectance of the milk relative to opal glass was measured at a series of nine wavelengths defined by filters in the range 400–700 mμ (Burton, 1956*b*). Changes in colour were determined by reflectance measurements with a single filter (no. 602) giving a mean wavelength of 470 mμ, and the results were expressed as a percentage of the value for an unheated control.

It is impossible to measure the reflectance of cow's milk after heating unless the milk has previously been homogenized, because rising of the fat interferes with the measurement. Unhomogenized goat's milk can, however, be heated and the reflectance measured without difficulty because of the small size of the fat globules. How-

ever, it is still desirable to homogenize goat's milk in practice if the milk is to be stored for more than 3–4 days, as a shallow cream layer will form. Therefore colour measurements were made of both unhomogenized and homogenized milk. The homogenization temperature was limited to 40 °C to avoid colour changes before the experimental heat treatment.

RESULTS AND DISCUSSION

The effect of heat treatment on the spectral variation of reflectance of goat's milk

The spectral variations of reflectance for raw, unhomogenized goat's milk and for the same milk heated for 90 and 225 min at 94·5 °C are shown in Fig. 1. The spectral variation for the unheated milk was found to be somewhat different from that

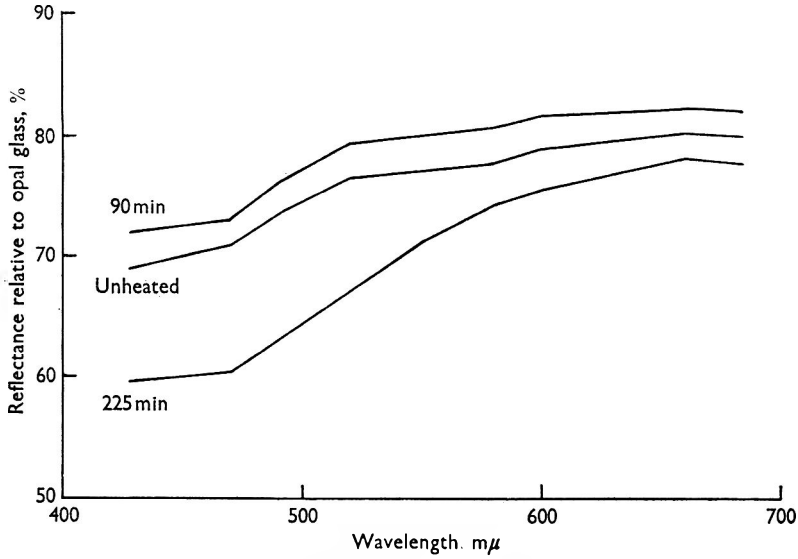

Fig. 1. Effect of heating at 94·5 °C on the spectral variation of reflectance of unhomogenized goat's milk.

previously reported for cow's milk. There was less change in the reflectance values throughout the spectrum, and in particular the high values found with cow's milk at wavelengths of about 600 mμ were not obtained with goat's milk. This is almost certainly because of the low carotene content of goat's milk.

When the milk was heated, the reflectance curves changed in the same way as the curves for cow's milk. Heating for 90 min caused an increase in reflectance throughout the spectrum, representing a whitening of the milk (Burton, 1955*a*). More severe heating for a time of 225 min caused a drop in reflectance throughout the spectrum, with the greater drop in the green and blue regions of the visible spectrum which constitutes browning.

The effect of temperature on the rate of browning

Fig. 2 shows the changes in the relative reflectance at a wavelength of 470 mμ for unhomogenized goat's milk heated for different times at temperatures between

approximately 94 and 120 °C. Curves for homogenized goat's milk heated for similar times and temperatures are shown in Fig. 3.

For each temperature there was an initial increase in reflectance representing whitening, followed by a decrease in reflectance which was sensibly linear over the range of heat treatments used. The rate of browning over the linear part of the curve may be calculated as the percentage decrease in reflectance for a 10 min interval.

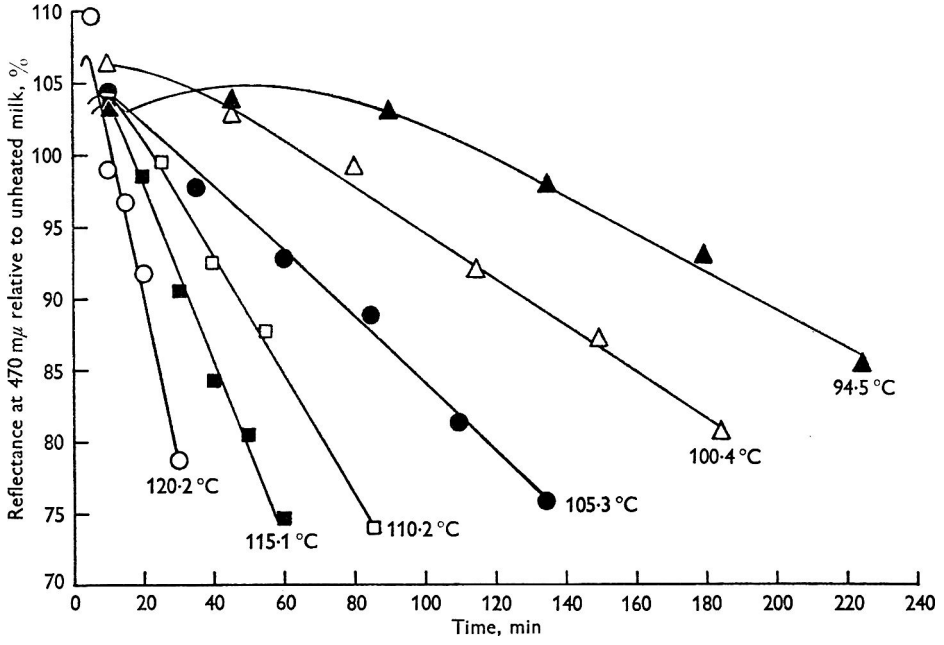

Fig. 2. Variation of relative reflectance with time of heating for unhomogenized goat's milk.

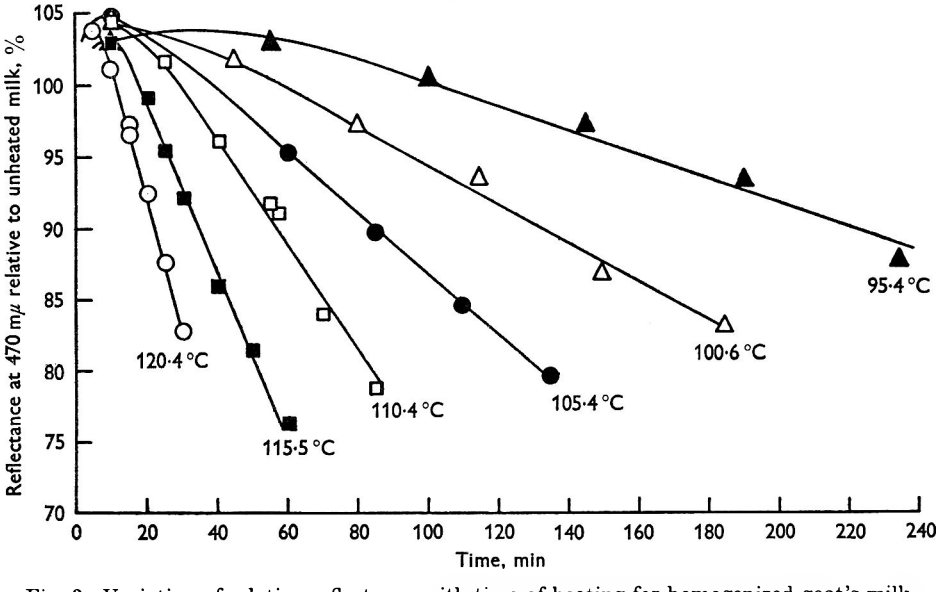

Fig. 3. Variation of relative reflectance with time of heating for homogenized goat's milk.

Fig. 4 shows the variations of the logarithm of the rate of browning with temperature for the homogenized and the unhomogenized samples.

The browning reaction for homogenized milk obeyed Arrhenius's Law, as shown by the good linear relationship in Fig. 4. The Q_{10} of the reaction was 2·6. The linear relationship was less good with unhomogenized milk, and showed some deviation at low temperatures. The Q_{10} for unhomogenized milk was 2·7. There was therefore no marked difference in the behaviour of homogenized and unhomogenized goat's milk. These Q_{10} values were lower than those for cow's milk, 2·9–3·1, determined by Burton (1954).

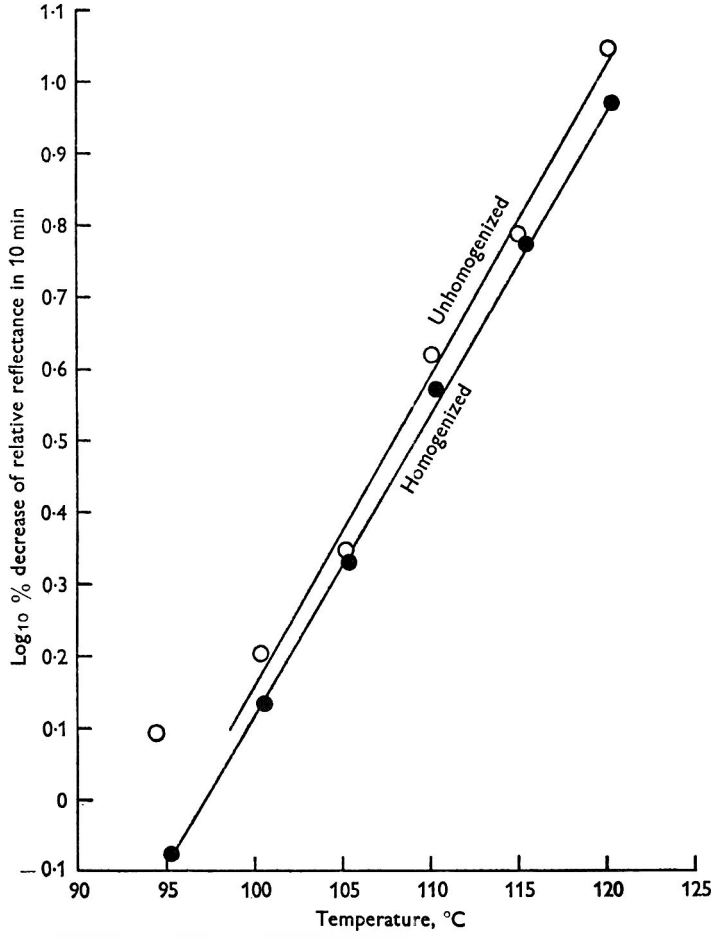

Fig. 4. Variation of rate of browning with temperature for unhomogenized and homogenized goat's milk.

The whitening of goat's milk

It is clear that goat's milk shows whitening, as evinced by a rise in reflectance over the whole visible spectrum, when it is heated to temperatures above about 75 °C. However, it proved unexpectedly difficult to produce curves relating rise in reflectance to time and temperature. For cow's milk it has been shown to be difficult to relate the amount of whitening to milk composition after a constant heat-treatment,

because of the wide variations obtained with apparently similar samples (Burton, 1955*b*). With goat's milk, wide variations in reflectance values were found for samples of the same milk heated at the same temperature. Typical whitening results are shown in Fig. 5 (*a*) and (*b*). The results for any one temperature were irregular, and it is difficult to construct a curve showing the variation of whitening with time for a constant temperature: the curve for one temperature tends to overlap the curves for others.

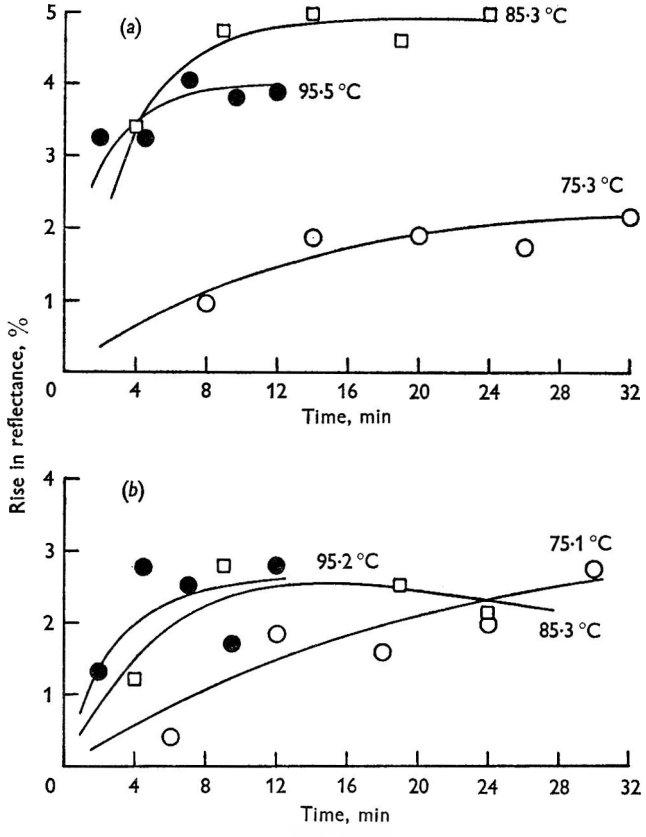

Fig. 5. The effect of time and temperature on the reflectance of a sample of homogenized goat's milk. (*a*) On day of milking, pH 6·63; (*b*) after 48 h at 5 °C, pH 6·48.

It has been suggested that the whitening of milk on heating is caused by the denaturation and subsequent aggregation of the soluble proteins (Burton, 1955*a*). The Aschaffenburg turbidity test for sterilized milk is based on the same phenomena, and Aschaffenburg (pers. comm.) found when applying this test to samples with faint residual turbidities that the amount of turbidity produced was highly sensitive to slight variations in the test procedure, e.g. in the amount of agitation of the sample. It is possible that the random variations in whitening found here arose from similar causes. Attempts to reduce the variations, by strict control of cooling of the sample and by avoiding unnecessary agitation of the milk, were not successful, however. Goat's milk appears to be more subject to these variations than

cow's milk, and the cause may lie in differences in the structure of the soluble protein.

In spite of variations, a general order of whitening can be found for any sample. It appears that the maximum amount of whitening in Fig. 5 (*a*) is about 5 % and that in Fig. 5 (*b*) is about 3 %. These two sets of results were obtained with the same sample of homogenized goat's milk measured (*a*) on the day of milking, and (*b*) 2 days later after storage at 5 °C. Similar results were obtained with unhomogenized milk. It seems, therefore, that in addition to being unusually sensitive to factors which affect the amount of whitening on heating, goat's milk when stored raw undergoes spontaneous changes which reduce the amount of whitening produced by subsequent heating. Changes in pH of the milk were too slight to have been responsible, as all the samples measured had pH values in the range 6·5–6·6. Protein changes may be the cause and further investigation may be worth while.

I express my thanks to Mr J. A. Pavey for his help with the experimental work.

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Printed in Great Britain

A quantitative study of changes in dried skim-milk and lactose-casein in the 'dry' state during storage

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(*Received* 13 *December* 1962)

Summary. Changes in dried skim-milk and in lactose-casein in the 'dry' state during storage at 45 °C and 75 % r.h. have been studied by the determination of free amino-N, ϵ-amino groups of lysine, galactose, lactulose, tagatose and 1-amino-1-deoxy-2-ketoses, and by the measurement of changes in colour, solubility and ferricyanide reducing power.

In both systems there is initially a close relationship between the formation of 1-amino-1-deoxy-2-ketoses and the decrease in free amino-N. This conforms with an Amadori rearrangement of an initially formed lactose-protein complex. Change in colour is rapid only after there has been a rapid formation of 1-amino-1-deoxy-2-ketoses and a corresponding decrease in free amino-N. This conforms with browning being due to a breakdown of the Amadori rearranged complex.

It is postulated that galactose and tagatose may be formed by both the base-catalysed degradation of lactose and also by breakdown of the Amadori rearranged lactose-protein complex. Lactulose is postulated to be formed only by base-catalysed degradation of lactose.

After an extensive study of the effect of storage on skim-milk powder Henry, Kon, Lea & White (1948) concluded that a major cause of deterioration is a reaction between the free amino groups of the milk protein, largely the ϵ-amino groups of lysine and the potential aldehyde group of lactose. They found that the reaction took place in two stages, the primary combination resulting in neither discoloration nor loss of solubility, these effects following only as a result of secondary changes which were at that time not fully understood. Their conclusions have been confirmed by the isolation of a sugar-protein complex from a reaction in the 'dry' state between glucose and casein (Lea & Hannan, 1950*a*). Because they failed to recover glucose from this complex on hydrolysis and because of its strong reducing power Lea & Hannan (1950*b*) proposed that the complex was not a simple N-glycoside, although such a substance may well be formed first, but is the product of isomerization as a result of some intramolecular change such as the Amadori rearrangement. Evidence that such changes do occur in heated milk has been provided by Adachi (1956), who isolated from the tryptic hydrolysate of the proteins of evaporated milk both amino acid glycosides with the structure of Schiffs bases, and also others with the structure

of N-substituted 1-amino-1-deoxy-2-ketoses. The latter presumably were formed by an Amadori rearrangement.

It is now generally considered that the main pathway for the deterioration of dried skim-milk during its storage is the formation of a protein-lactose complex and its Amadori rearrangement. However, the mechanism of the formation of lactose degradation products such as galactose, lactulose and tagatose which have been found in stored dried skim-milk (Richards & Chandrasekhara, 1960) is open to speculation.

In this paper an attempt has been made to gain a better understanding of the mechanisms of deterioration of dried skim-milk and 'dry' lactose-casein and of the formation of lactose degradation products in these systems by following, quantitatively, many of the changes associated with browning.

EXPERIMENTAL

Dried skim-milk. Fresh, unheated cow's milk was warmed to 37 °C and the fat removed by centrifugal separation. The skim-milk (6 l) was freeze dried and the product powdered, mixed and then immediately placed in storage containers.

Lactose-casein. Casein was prepared from fresh unheated cow's milk (5 l), after centrifugal separation of the cream, by adding 0·1 N-HCl slowly with stirring until the pH dropped to 4·6. The precipitate was allowed to settle and the whey was then removed by siphoning. The precipitate was washed five times by stirring it with distilled water (3 l portions), allowing the precipitate to settle and then removing the wash-water by siphoning. The casein was suspended in water (4 l) and the pH of the suspension adjusted to 6·3 by adding 0·1 N-NaOH slowly with stirring. As the casein dissolved the pH dropped and was adjusted to 6·3 by the addition of further 0·1 N-NaOH. When the casein was completely dissolved the pH was finally adjusted to 6·3 and lactose (230 g) was added and dissolved by stirring. The solution was filtered and then freeze-dried. The lactose-casein thus prepared consisted of white glistening flakes which were powdered, mixed and then immediately placed in storage containers.

Storage. The dried skim-milk and lactose-casein were stored at 45° C in airtight jars partly filled with a saturated solution of sodium chloride giving a relative humidity of 75 %. The materials were held in thimbles of Whatman 3 MM paper (9 cm diameter × 18 cm) which were placed on glass stands so as to be above the salt solution.

Measurement of colour. Samples of the stored materials were ground and sieved and the fraction passing a 30-mesh test sieve (B.S. 410: 1943 for sieves) but held on a 60-mesh test sieve was examined in an EEL reflectance spectrophotometer using magnesium carbonate as a standard white surface. Colour has been reported as the reciprocal of percentage full-scale deflexion using filter 601 (4250 Å).

Solubility. The standard method of the American Dry Milk Institute (1954) was used to determine solubility at 37 °C.

Estimation of free amino groups. Free amino-N was determined by the manometric method of Van Slyke according to the procedure described by Lea (1948) using a reaction time of 30 min at 25 °C.

Estimation of free ε-amino groups of lysine. Free ε-amino groups of lysine were determined by the method of Carpenter (1960) modified by replacing stage 3 (extraction with methoxycarbonyl chloride) with a chromatographic separation of ε-DNP-lysine (Baliga, Bayliss & Lyman, 1959). The eluate from the area of the paper chromatogram containing ε-DNP-lysine was made up to 25 ml in a volumetric flask with N-HCl and its extinction coefficient was read at 435 mμ in a Beckman model DU spectrophotometer. The concentration of ε-DNP-lysine was then read from a standard curve that had been prepared using ε-DNP-lysine hydrochloride synthesized by the method of Porter & Sanger (1948).

Estimation of carbohydrates

1. *Extraction.* Samples (10 g) were stirred at 37 °C with water (40 ml) for 30 min and then 95% ethanol (170 ml) also at 37 °C was added with further stirring. After filtration any residue was extracted twice more using the same procedure. The combined extracts were then concentrated under vacuum and made up to 25 ml in a volumetric flask with distilled water.

2. *Chromatographic separation.* Portions of the extracts (0·4 ml) were applied as narrow bands (10 cm long) to acid washed Whatman 3 MM paper and chromatographed for 24 h using the organic phase of ethyl acetate–acetic acid–water (3:1:1) as the developing solvent. The papers were dried for 24 h at room temperature and then guide strips were sprayed with *p*-anisidine hydrochloride reagent and heated. The areas of the main chromatogram corresponding to each sugar in the guide strip were eluted and the volume of each eluate adjusted to 10 ml. For each sugar a blank portion of similar area from the same chromatogram was eluted to provide a blank.

3. *Estimation.* Galactose was estimated in the appropriate eluates by both the method of Nelson (1944) and that of Bath (1958). Tagatose and lactulose were estimated by Bell's (1955) resorcinol method for ketoses. Standard curves were prepared using crystalline D-tagatose (L. Light and Co. Ltd.) and D-lactulose prepared by the method of Montgomery & Hudson (1930) modified in that ion exchange resins were used to remove inorganic compounds.

Ferricyanide reducing value. Samples (1 g) were mixed with water (30 ml) at 20 °C in a Waring blendor and the reducing value of this suspension determined by the Chapman–McFarlane method for the estimation of acid ferricyanide reducing groups as modified by Choi, Koncus, Cherrey & Remaley (1953).

1-Amino-1-deoxy-2-ketoses. These were estimated by the method of Keeney & Bassette (1959) which depends upon the digestion of the sample under optimum conditions for the production of 5-hydroxymethylfurfural from 1-amino-1-deoxy-2-ketoses without causing primary degradation of ketose to 5-hydroxymethylfurfural.

Protein-lactose complex. A sample of dried skim-milk (5 g) which had been stored for 22 days, and a sample of lactose-casein (10 g) which had been stored for 10 days, were mixed with water, transferred to narrow (3 cm diameter) Cellophane sacs and dialysed for several days against many changes of distilled water. When free sugars could no longer be detected in the liquid in the dialysis sacs by paper chromatography, the dialysed materials were freeze dried and stored again under the conditions previously

detailed. The complexes were examined chromatographically for sugars after storage for 10 and 31 days and quantitatively for free 5-hydroxymethylfurfural (Keeney & Bassette, 1959) and changes in colour after storage for 31 days.

RESULTS

Changes in colour

The development of a brown colour with time in both dried skim-milk and lactose-casein is shown in Fig. 1. It can be seen that while the casein-lactose was appreciably lighter in colour than the dried skim-milk before storage, it developed a brown colour more rapidly. In both systems the rate of discoloration was roughly linear over most of the storage period (cf. Henry *et al.* 1948). Lea & Hannan (1949) found in their study of casein-glucose that a marked lag period preceded the development of colour. Such a lag is not clear in Fig. 1 but the matter is discussed later (p. 231).

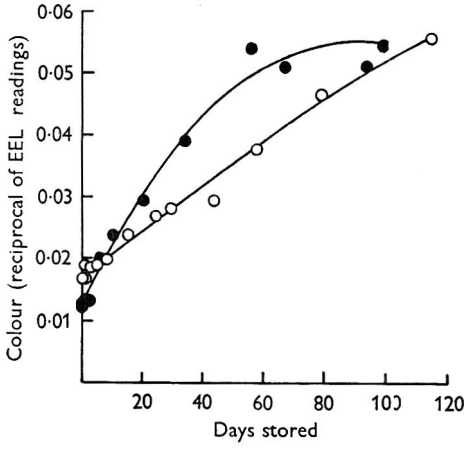

Fig. 1

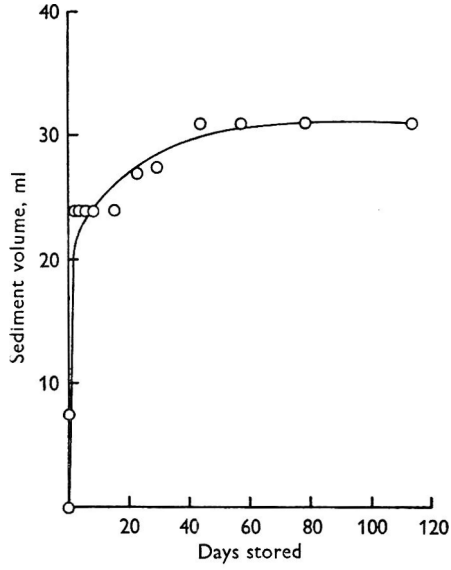

Fig. 2

Fig. 1. Changes in colour of dried skim-milk (○) and lactose-casein (●) in the 'dry' state during storage at 45 °C and 75 % r.h.

Fig. 2. Change in solubility of dried skim-milk stored at 45 °C and 75 % r.h.

Changes in solubility

The effect of storage at 45 °C and 75 % r.h. on the solubility measured at 37 °C of dried skim-milk is shown in Fig. 2. There was no lag in the onset of loss of solubility as was found for skim-milk powder stored at 20, 28·5 and 37 °C by Henry *et al.* (1948). The lactose-casein system behaved similarly to the glucose-casein complex studied by Lea & Hannan (1949) in that it had a definite affinity for water even when markedly coloured. After storage it formed a gel rather than a true solution and this made it difficult to decide when insolubility began. For this reason no results are reported.

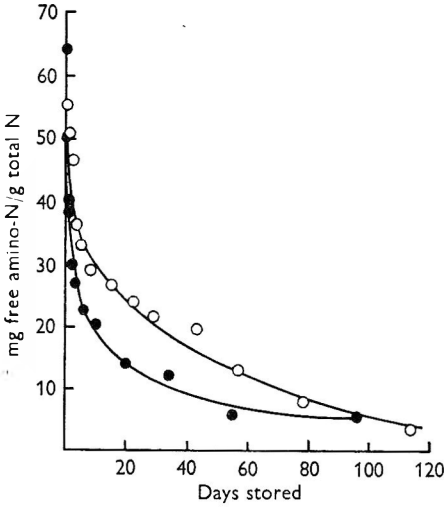

Fig. 3

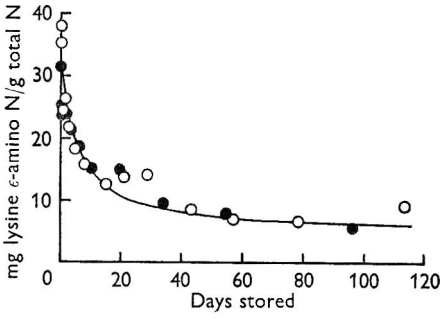

Fig. 4

Fig. 3. Loss of free amino nitrogen by the protein of dried skim-milk (○) and 'dry' lactose-casein (●) during storage at 45 °C and 75 % r.h.

Fig. 4. Loss of lysine ε-amino groups by the protein of dried skim-milk (○) and 'dry' lactose-casein (●) during storage at 45 °C and 75 % r.h.

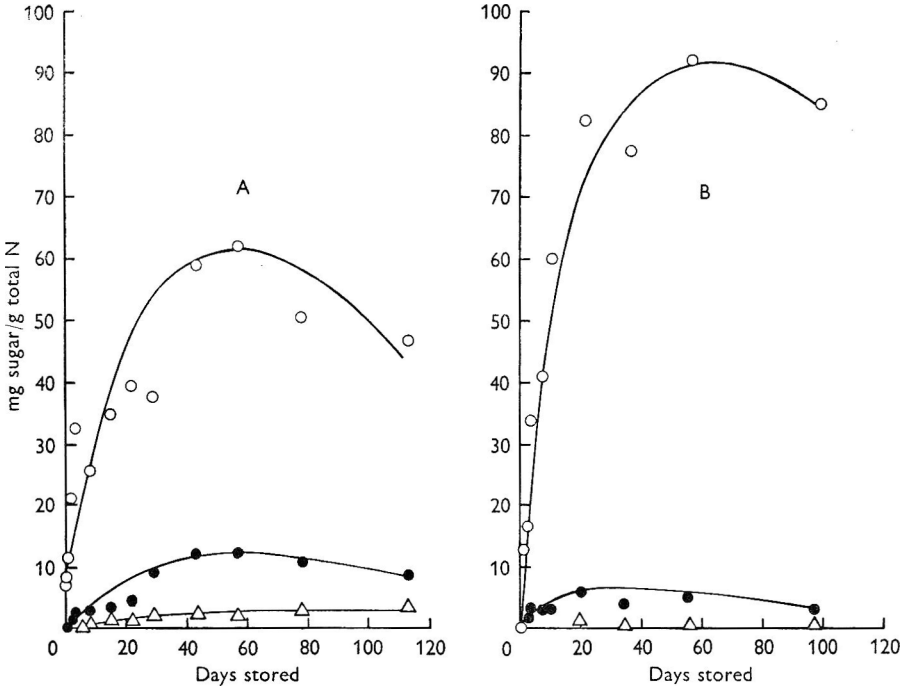

Fig. 5. Production of galactose (○), lactulose (●) and tagatose (△) during the storage of A, dried skim-milk and B, 'dry' lactose-casein at 45 °C and 75 % r.h.

Changes in free amino groups

The results summarized in Fig. 3 show the very rapid loss in the free amino groups of the protein that occurred during the storage of both skim-milk powder and lactose-casein. In both systems more than 50 % of the free amino groups originally present were modified in the first 10 days of storage.

Changes in free ε-amino groups of lysine

The method used gave the expected results when applied to sodium caseinate (51 mg/g protein N) and gelatin (28 mg/g protein N) but did not do so for dried skim-milk or lactose-casein even when allowances were made by recovery trials for loss of

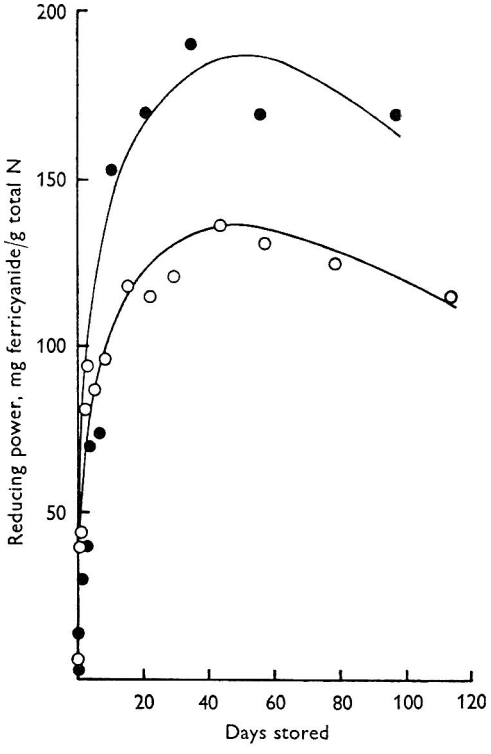

Fig. 6

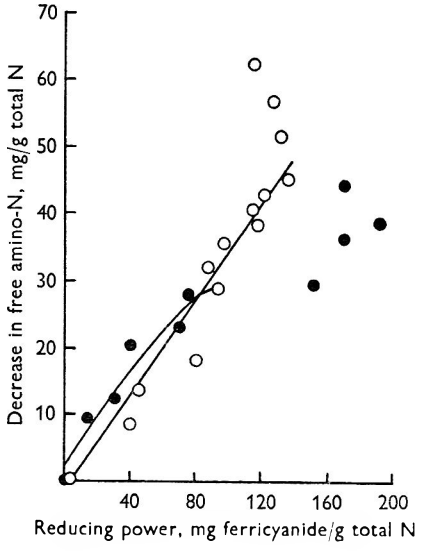

Fig. 7

Fig. 6. Increase in the reducing power of dried skim-milk (○) and 'dry' lactose-casein (●) during storage at 45 °C and 75 % r.h.

Fig. 7. Relationship between increase in reducing power and loss of free amino-N in dried skim-milk (○) and 'dry' lactose-casein (●) during storage.

ε-DNP-lysine during the hydrolysis stage of the determination. However, since the values obtained were reproducible under the standard experimental conditions used they are reported here in Fig. 4. The determination of ε-amino groups of lysine in the presence of carbohydrate is being further investigated.

Changes in carbohydrates

The changes in the levels of galactose, lactulose and tagatose are shown in Fig. 5. Galactose was present in the dried skim-milk before storage but in the lactose-casein there was a 24-h delay before it was detectable. In both systems there was a rapid increase in the galactose content until a maximum was reached after about 50 days' storage. There was also a delay before lactulose and tagatose could be detected. As can be seen in Fig. 5 the concentration of these sugars was at all times much lower than that of galactose.

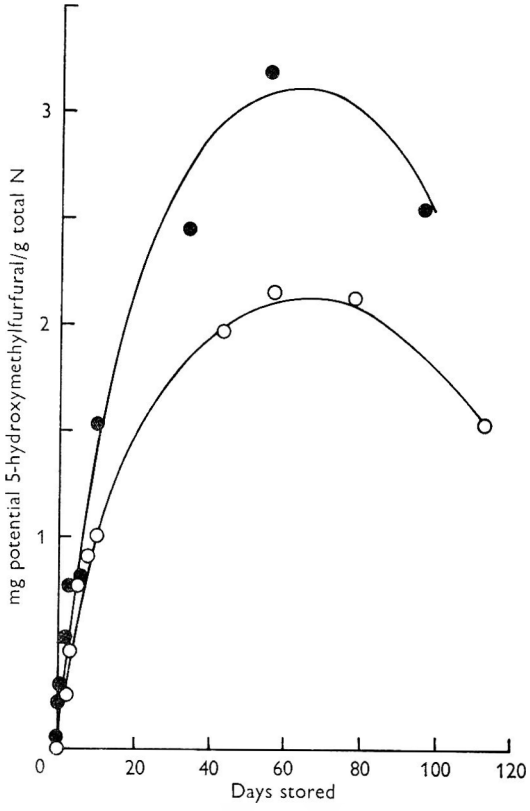

Fig. 8. Formation of 1-amino-1-deoxy-2-ketoses during the storage of dried skim-milk (○) and 'dry' lactose-casein (●) at 45 °C and 75% r.h.

Changes in ferricyanide reducing power

The results summarized in Fig. 6 show that as with many of the other changes studied in these experiments there was an initial rapid increase in reducing power which reached a maximum after 50–60 days of storage and then declined. Henry *et al.* (1948) showed that the increase in reducing power towards ferricyanide, as measured by the Chapman & McFarlane method, correlated well with the progress of the primary sugar-amino reaction until about half of the total free amino nitrogen had been complexed. Fig. 7, in which reducing power towards ferricyanide is plotted against free amino nitrogen, confirms this relationship in dried skim-milk and shows that it also holds for lactose-casein.

Changes in 1-amino-1-deoxy-2-ketoses

The results are summarized in Fig. 8 where it can be seen that again there was an initial rapid increase towards a maximum value which was reached after about 50–60 days.

Changes in protein-lactose complex on storage

No sugars could be found by paper chromatography in the protein-lactose complexes obtained by dialysis of stored dried skim-milk and lactose-casein when such complexes had been stored for a further 10 days. However after 31 days' storage galactose was readily identified chromatographically but lactulose was not. During the further storage of 31 days the colour of the complex from dried skim-milk increased from 0·033 to 0·042 units and that from lactose-casein from 0·025 to 0·033 units. Free 5-hydroxymethylfurfural was formed in both the former (0·058 mg/g) and the latter (0·023 mg/g).

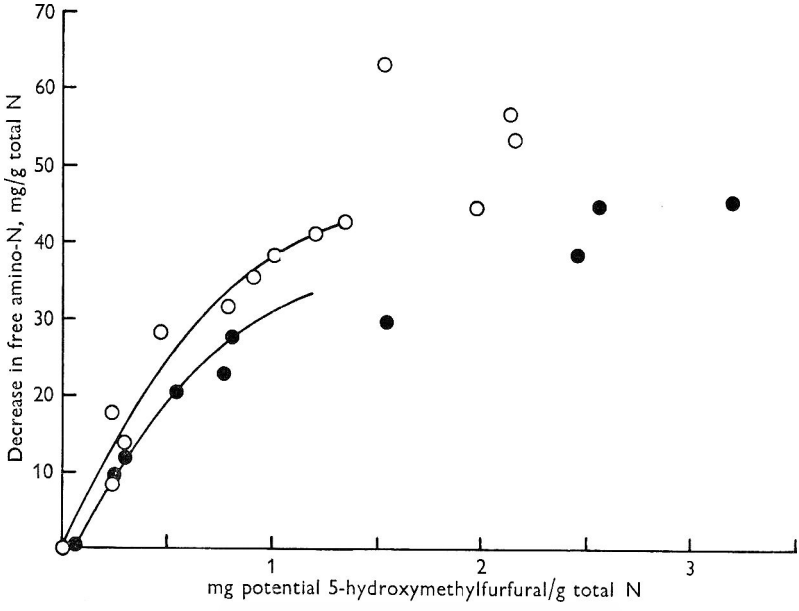

Fig. 9. Relationship between the formation of 1-amino-1-deoxy-2-ketoses and the decrease in free amino-N in dried skim-milk (○) and 'dry' lactose-casein (●) during storage.

DISCUSSION

Gottschalk (1952) found that the outstanding property of 1-amino-1-deoxy-2-ketoses was the ease with which they form 5-hydroxymethylfurfural upon mild acid treatment, and since even the acid labile D-fructose does not yield appreciable amounts of 5-hydroxymethylfurfural under the same conditions he proposed that the production of the latter compound by dilute acid may be used as a tracer for 1-amino-1-deoxy-2-ketoses. Keeney & Bassette (1959) made use of this in their determination of 'potential' 5-hydroxymethylfurfural which they realized was being formed from 1-amino-1-deoxy-2-ketoses under the digestion conditions they used. In this paper

then 'potential' hydroxymethylfurfural is being used as a measure of 1-amino-1-deoxy-2-ketoses. Fig. 9 shows that an almost linear relationship existed between the formation of 1-amino-1-deoxy-2-ketoses and the decrease that occurred in amino nitrogen in both dried skim-milk and lactose casein until approximately half of the free amino nitrogen had reacted. This is consistent with the rapid conversion by an Amadori rearrangement of an amino-sugar complex into a 1-amino-1-deoxy-2-ketose. At that stage of the reaction, that is until about half of the free amino-N had reacted, there

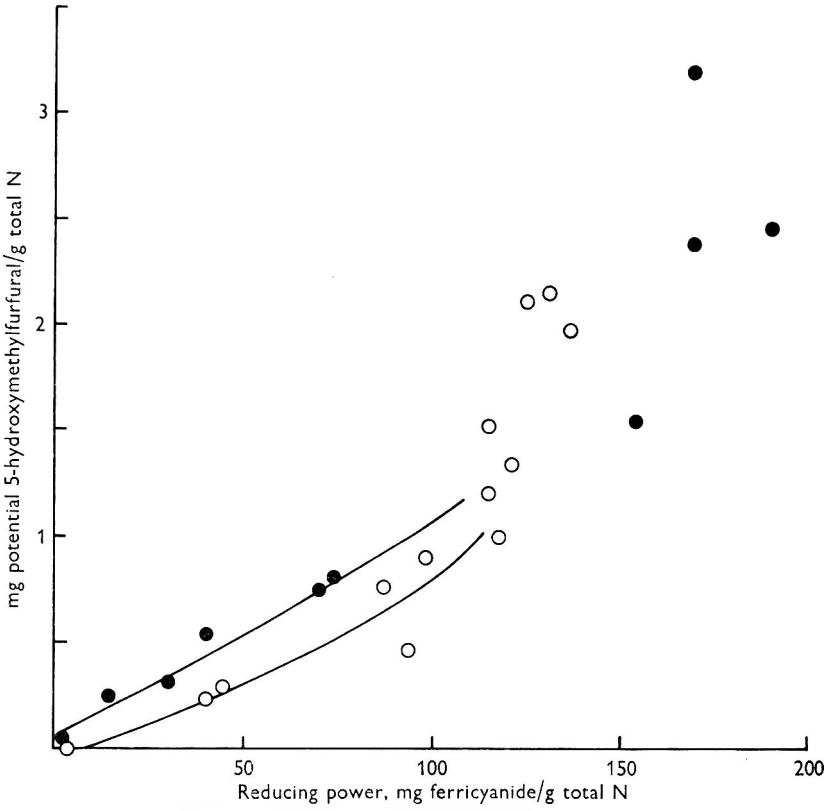

Fig. 10. Relationship between the formation of 1-amino-1-deoxy-2-ketoses and the increase in reducing power in dried skim-milk (○) and 'dry' lactose-casein (●) during storage.

was also a close relationship (Fig. 10) between the formation of the 1-amino-1-deoxy-2-ketose complex and the reducing power of the product towards ferricyanide. This suggests that the reducing power is due to the 1-amino-1-deoxy-2-ketoses which are known to have very strong reducing power (Gottschalk, 1952; Hodge, 1953). During this initial stage of the reaction, which lasted about 10 days, there was very little change of colour. This is not clear in Fig. 1 but becomes more obvious when change of colour is plotted against decrease in amino nitrogen (Fig. 11). From the figures it can be seen that about half of the amino nitrogen was complexed before any marked change of colour occurred.

Two mechanisms have been proposed to explain the formation of lactulose, galactose and tagatose from lactose in heated or stored milk products. On the one hand

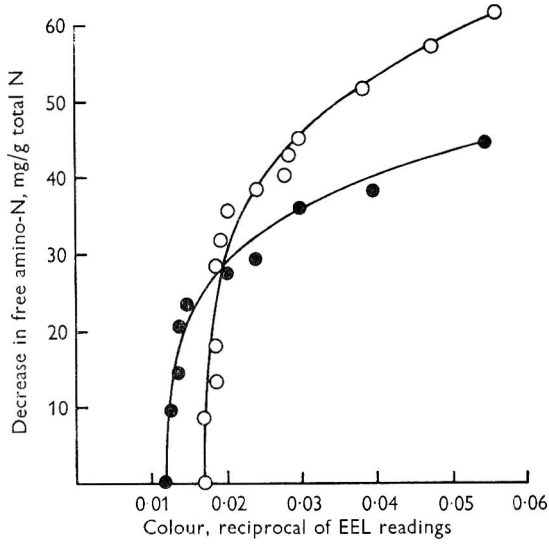

Fig. 11. Relationship between colour and loss of free amino-N in dried skim-milk (○) and 'dry' lactose-casein (●) during storage.

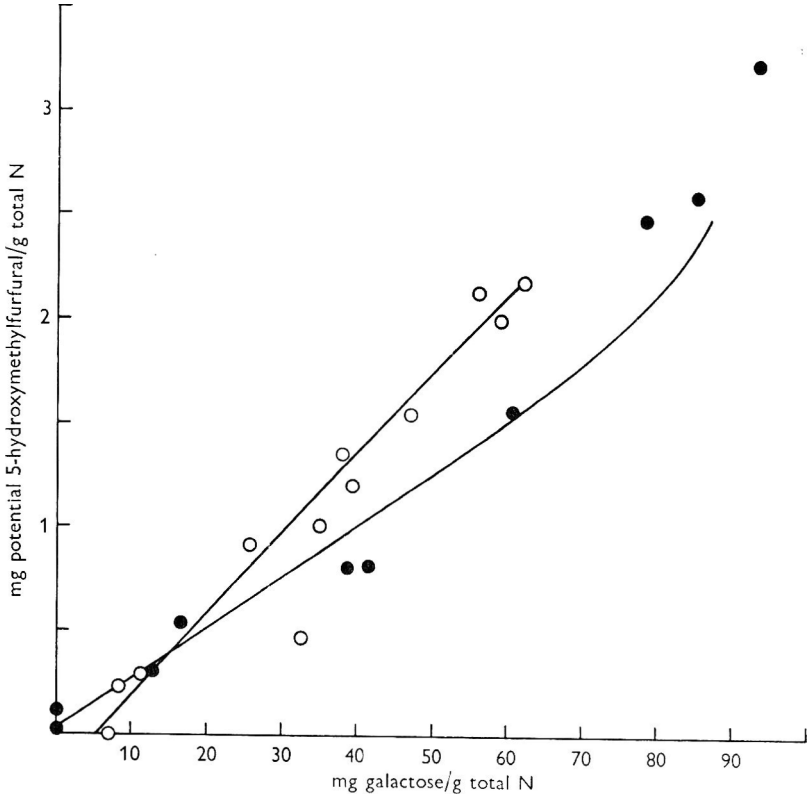

Fig. 12. Relationship between changes in galactose and 1-amino-1-deoxy-2-ketose content of dried skim-milk (○) and lactose-casein (●) during storage.

it has been suggested (Richards & Chandrasekhara, 1960) that while the protein-sugar reaction is responsible for most of the browning of dried milk during storage, lactulose, galactose and tagatose are formed by a degradation of lactose catalysed by the free basic amino groups of casein in a reaction sequence similar to that proposed by Corbett & Kenner (1953) for the degradation of lactose by strong alkali. On the other hand Adachi & Patton (1961) assume that lactulose is formed by the hydrolytic degradation of the Amadori rearrangement product of lactose-casein. In apparent conflict with this latter theory is the observation that an outstanding feature of most 1-amino-1-deoxy-2-ketoses so far studied is that the sugar-amino linkage is no longer readily susceptible to hydrolysis (Hodge, 1955) but rather is broken by a dehydration and fission of the sugar moiety. An exception to this is *p*-tolyl-N-isolactosylamine in the acid hydrolysate of which Adachi (1957) identified lactulose. The properties of the protein-sugar complexes are of interest in helping to clarify the route or routes by which galactose and lactulose are formed. On further storage these complexes formed galactose and 5-hydroxymethylfurfural but not lactulose. This conforms with the complexes being 1-amino-1-deoxy-2-ketoses formed by an Amadori rearrangement, since during the breakdown of such compounds the ketose moiety attached to the protein is dehydrated to 5-hydroxymethylfurfural and galactose is liberated by rupture of the disaccharide. It would seem then that galactose can be formed both by the base catalysed degradation of lactose and also by the breakdown of the protein-sugar complexes. This could explain why the concentration of galactose in the stored samples is greater than that of lactulose, in contrast to the observation of Corbett & Kenner (1953) that during the degradation of lactose by lime water, lactulose could be clearly detected before galactose and remained at a greater intensity throughout the reaction. Lactulose, however, is not formed via the protein-sugar complex and presumably is formed only by the base catalysed degradation of lactose. Fig. 12 shows what appears to be a close relationship between the concentration of galactose in the stored materials and that of 1-amino-1-deoxy-2-ketoses. This can be explained by postulating that while galactose can be formed by both mechanisms it is in fact formed mainly by the breakdown of 1-amino-1-deoxy-2-ketoses.

The author thanks Miss B. Handley for technical assistance.

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Printed in Great Britain

Hydrogen sulphide in Cheddar cheese; its estimation and possible contribution to flavour

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(*Received* 31 *December* 1962)

Summary. A modified entrainment method is described for the estimation of micro-quantities of hydrogen sulphide in cheese. Optimum recovery is only possible if the conditions are very acid, if oxygen is excluded and if appreciable loss by diffusion is avoided by minimizing the surface area of the sample and the time it is exposed to the atmosphere. The reproducibility of determinations from the same cheese was satisfactory.

The concentration of H_2S in New Zealand Cheddar cheese curd at hooping was less than 20 μg/100 g but increased after ripening for 1–4 months to an average concentration of 55 μg, thereafter remaining fairly constant. There appeared to be no simple relationship between characteristic Cheddar flavour and H_2S concentration.

In some recent investigations of the chemical compounds responsible for the characteristic Cheddar flavour attention has centred on the contribution of H_2S. Kristoffersen & Gould (1960) found a significant statistical correlation between H_2S and Cheddar flavour, in particular between the characteristic flavour and the relative concentrations of free fatty acids and H_2S. Kristoffersen (1961), however, subsequently reported that the flavour sensation provided by some Cheddar cheeses was not always related to the level of H_2S particularly in the early stages of curing. Walker (1961) attempted to simulate a Cheddar type flavour by the addition of mixtures of methyl ketones and fatty acids to bland cheese curd, but found that the flavours produced were sharp and musty unless thioacetamide, a source of H_2S, was added, in which case the flavour was much improved.

In the course of investigations in this laboratory into the chemical nature of the substances responsible for Cheddar flavour an attempt was made to check these observations. It is generally accepted that low concentrations of H_2S are best determined quantitatively by the method of Almy (1925), and the conditions necessary for the optimum absorption of H_2S and its conversion to methylene blue have been thoroughly investigated (Sands, 1949; Fogo & Popowsky, 1949; Dale & Davies, 1951; Marbach & Doty, 1956). Some workers have used steam distillation to determine the H_2S in cheese, but, as sulphide is known to be liberated on heating proteinaceous foods (e.g. Pippen & Eyring, 1957; Townley & Gould, 1943), such recovery methods were not considered suitable. The only method which does not use steam distillation is that of Kristoffersen, Gould & Harper (1959). They blended the cheese in sodium

citrate solution, transferred the blend to a separate aerating vessel, acidified with HCl and then passed air through the blend. The H_2S was trapped in zinc acetate solution and subsequently converted to methylene blue. This communication describes a modification of their method and its use in an investigation of the H_2S concentration in New Zealand cheeses throughout ripening.

METHODS

The cheeses used in these investigations were made and ripened in the Institute's factory using normal New Zealand commercial methods. All cheeses used in preliminary experiments were 6–9 months old and of good Cheddar flavour. They were lettered A to F for purposes of identification. In subsequent work, sixteen cheeses (details given later) were examined at intervals during ripening to ascertain the effect of different starter organisms and of other bacteria on H_2S production.

In preliminary exploratory experiments the original method of Kristoffersen *et al.* (1959) was used. However, poor recoveries (about 50 %) of H_2S added to the cheese before blending and erratic results with samples from the same cheese made it clear that Kristoffersen's method led to loss of H_2S during the blending. The apparatus was therefore modified by sealing the blender jar so that it could be used as the vessel from which the H_2S was removed from the cheese emulsion. Oxygen-free nitrogen instead of air was used for entraining the H_2S which was subsequently absorbed in zinc acetate solution.

Table 1. *The effect on the recovery of hydrogen sulphide of blending cheeses under different conditions*

Cheese	Blending time, min	Atmosphere	Type of distilled water	H_2S concentration, μg/100 g
A	0·5	Air	Ordinary	38
	2	Air	Ordinary	27
	4	Air	Ordinary	26
B	0·5	Air	Ordinary	30
	4	Air	Ordinary	24
	10	Air	Ordinary	20
	20	Nitrogen*	Ordinary	36
	20	Nitrogen*	Freshly boiled	53
C	4	Air	Ordinary	31
	4	Air	Freshly boiled	36
	20	Nitrogen*	Ordinary	52
	20	Nitrogen*	Freshly boiled	65

* Homogenized in sealed blender and hydrogen sulphide removed without transferring sample to another vessel. Remaining samples homogenized in open air and transferred to a separate aerating vessel for removal of hydrogen sulphide as in original method of Kristoffersen *et al.* (1959).

Table 1 gives results obtained with cheese samples treated in various ways. Experiments in which Kristoffersen's method (cheeses B and C, 4 min) was compared with the modified method (cheeses B and C, 20 min) disclosed that losses of H_2S took place through diffusion and oxidation during the preparation of cheese emulsion in an open vessel. It is evident from the figures that there was a progressive loss of H_2S from the cheese as the time of blending in air was extended, and that the use of ordinary distilled water instead of freshly boiled water led to even lower results,

possibly due to oxidation. The total losses could, under the worst conditions, be of the order of 50 %. The claim of Kristoffersen *et al.* (1959) that recovery of added H_2S was quantitative was apparently based on trials in which the H_2S was added to the final extraction apparatus after the preliminary blending of the cheese—a stage at which diffusion losses could no longer occur.

It would be difficult to determine how much H_2S escapes from a plug of cheese from the moment of drawing until it is transferred to a blender, but it is to be expected that the loss would be related to the surface area exposed. Experiments in which the cheese was grated in an atmosphere of nitrogen (for a period of 15 min immediately before analysis) for comparison with plugs exposed for 15 min in nitrogen or in air (Table 2) showed that approximately 50 % of the H_2S could be lost by oxidation or by diffusion from the large surface created by grating. However, the absence of a significant difference between the plugs drawn in air and nitrogen, respectively, indicates that, provided the plugs are not broken up, oxidation does not seriously affect recovery of H_2S and that it is therefore unnecessary in a routine examination to draw plugs anaerobically.

Table 2. *Variation in hydrogen sulphide* (μg/50 g) *found in cheese exposed for* 15 *min in nitrogen or air, before analysis in a sealed blender*

	In nitrogen		In air
Experiment	Grated	Whole plugs	Whole plugs
1	17	36	45
2	18	31	37
3	22	35	32
4	19	35	42
5	20	41	36
Mean	19	36	38

One further modification of Kristoffersen's method was the use of phosphoric acid as both the emulsifying and acidifying agent in place of sodium citrate and hydrochloric acid. Phosphoric acid had no measurable effect upon cysteine, cystine or glutathione whereas hydrochloric acid liberated limited quantities of H_2S from these compounds. Neither of these acids liberated H_2S from methionine, thiamine, casein, lactalbumin or cheese fat. In view of its greater selectivity, phosphoric acid was used in all the later experiments.

Numerous experiments showed that the addition of 100 ml of phosphoric acid to 100 g of cheese resulted in 100 % recovery (± 10 %) of known concentrations of H_2S added to the slurry. The use of less than 100 ml of phosphoric acid, under the conditions of the estimation, consistently gave slightly less satisfactory recoveries (80–100 %). It should be stressed, however, that the ready oxidation of very dilute solutions of H_2S noted by Dale & Davies (1951) and Marbach & Doty (1956) made difficult the accurate determination of the extent of the recovery.

Method of estimation finally adopted

The apparatus consisted of a glass blender jar attached directly to boiling tubes containing the trapping solution. The glass blender jar was sealed with a large rubber bung which had been boiled in strong aqueous NaOH solution and whose inner surface

was coated with silicone grease prior to each estimation. Trial runs showed the apparatus to be free from sulphide.

A plug was drawn from the cheese with a butter trier and 100 g were rapidly weighed and covered with 250 ml of freshly boiled distilled water in the blender. Special care was taken to avoid breaking the plug prior to blending. Oxygen-free nitrogen was passed through the apparatus, 5 min being allowed to free the blender and trapping tubes from air before blending started. Then 100 ml of oxygen-free A.R. phosphoric acid (sp.gr. 1·75) was added and the cheese blended for 20 min. The H_2S entrained in the nitrogen was absorbed in 42 ml of 1 % (w/v) zinc acetate solution and 2 ml of 10 % (w/v) NaOH solution. Methylene blue was subsequently developed by the addition of 5 ml of *p*-diamino-NN-dimethylaniline hydrochloride (1 g/l of approx. 5 N-HCl) and 1 ml of 1 % (w/v) $FeCl_3$ solution. The intensity of the colour was read on a Beckman spectrophotometer at 678 mμ 1 h after the addition of reagents, and the concentration of H_2S was determined from a calibration curve prepared as described by Brenner, Owades & Golyzniak (1953).

The reproducibility of the technique was assessed by analysing replicate samples from each of two cheeses. The mean concentration of H_2S in cheese E was 53·0 μg/100 g, S.D. 5·0 (twenty-one samples). For cheese F (fifteen samples) the mean concentration was 52·0 μg/100 g, S.D. 5·0.

RESULTS

Variation in concentration of hydrogen sulphide during making and ripening

To determine whether the H_2S found in cheese was due solely to microbial activity or was derived partly from milk, the concentration of H_2S was determined during making and ripening. In contrast to the findings of Townley & Gould (1943), who reported no appreciable sulphide in milk heated to a temperature of 76 °C, pasteurized milk (71 °C for 15 sec) was found to contain reasonably high concentrations of H_2S (Table 3). Part of the sulphide in pasteurized milk was lost either by diffusion or oxidation during the cheese-making process, that which remained being concentrated in the curd, the whey containing very little.

Table 3. *Concentration of hydrogen sulphide in pasteurized milk, whey, salted curd and mature cheese*

	No. of batches examined	Concentration of H_2S
Pasteurized milk	8	20–45 μg/l
Whey	6	3–5 μg/l
Salted curd	16	8–20 μg/100 g
Mature cheeses, 6–9 months old	25	45–80 μg/100 g

Since the concentration of H_2S increased during ripening it seemed likely that bacteria were responsible for its formation. Cheeses were therefore made from milk to which bacteria thought to be of possible importance in the development of the characteristic Cheddar flavour had been added.

Addition of bacterial cultures to milk prior to cheese-making

Four cheeses were made with *Streptococcus cremoris*, strain HP, as starter. No additional strains were added to the milk from which the first cheese was made. To the milk for the second was added a 0·1 % inoculum of a yeast milk culture of a strain of micrococcus, L_1 (Robertson & Perry, 1961), and to the milk for the third a 1 % inoculum of a yeast milk culture of *Lactobacillus plantarum-casei*, strain 25·2 (Sherwood, 1939). To the milk for the fourth cheese the same inocula of both strain L_1 and

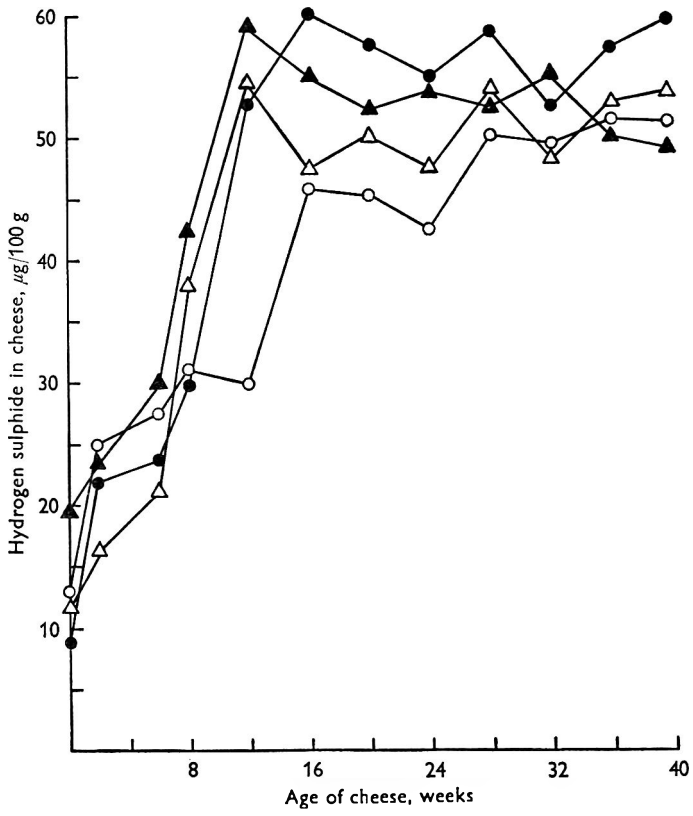

Fig. 1. Production of hydrogen sulphide during ripening of Cheddar cheese made with single-strain starters, *Str. cremoris* (HP, ○–○; Tr, ●—●) and *Str. lactis* (ML_2, △—△; ML_3, ▲—▲). Each point represents the average concentration in duplicate samples each of 100 g of cheese.

strain 25·2 were added. In a similar way, four cheeses were made with *Str. cremoris*, strain TR, and four each with two strains of *Str. lactis* (ML_2 and ML_3). These sixteen cheeses were examined at monthly intervals for flavour and H_2S concentration. The flavours varied from mild to strong Cheddar after 6 months and, in addition, cheeses made with *Str. lactis* had definite flavour defects, mainly described as 'fruity' or 'lactis' (Perry, 1961). In every cheese the concentration of H_2S reached a maximum of between 45 and 60 μg/100 g after 1–4 months and thereafter remained fairly constant.

Fig. 1 shows the changes in concentration of H_2S content during ripening of the

four cheeses made with the four different single-strain starters. There were considerable differences in intensity of Cheddar flavour between the four cheeses but a marked similarity in the pattern of H_2S production. The addition of cultures of a lactobacillus and a micrococcus to the cheese milk did not, in these experiments, enhance the cheese flavour to any great extent. The four cheeses made with starter and an added inoculum of the lactobacillus reached their H_2S maxima before the other twelve cheeses (i.e. after 1–2 months' ripening) but otherwise the additions had no appreciable effect upon the production of H_2S which in all cases followed the general pattern shown in Fig. 1. There appeared to be no simple relationship between characteristic Cheddar flavour and the H_2S concentration.

DISCUSSION

A distinct 'sulphide' smell is sometimes momentarily encountered when mature cheeses are cut and presumably this is due to the escape of free H_2S, a loss which would be difficult to avoid. The flavour of cheese when eaten will depend only upon the H_2S remaining in the cheese. It would seem reasonable, therefore, to attempt to correlate the flavour of cheese with the concentration of H_2S.

It was found that the concentration of H_2S increased until the cheeses were from 1 to 4 months old, and then remained fairly constant. High concentrations of H_2S have a toxic effect upon many bacterial species and it is possible that the species producing the H_2S in Cheddar cheese may build up a toxic concentration and cease to grow. Alternatively the H_2S may be formed by organisms, such as the starter bacteria or micrococci, which decline rapidly in numbers in the first few weeks after manufacture leading to a corresponding decrease in H_2S production.

In the early stages of ripening, both the H_2S concentration and the intensity of cheese flavour increased. However, while the Cheddar flavour of the cheese continued to intensify, the H_2S concentration remained more or less constant. Similarly, although the typical Cheddar flavour of sixteen cheeses sampled over a period of 9 months varied markedly in intensity, the quantities of H_2S found (45–60 μg/100 g) in all the mature cheeses were not greatly different. The contribution of H_2S to flavour would not therefore appear to be direct in the sense that an increase in H_2S content is necessarily associated with an increase in typical Cheddar flavour. It may, however, be that the H_2S combines with some other product of ripening, perhaps with carbonyl, carboxyl or ester groupings, with which it is known to form addition compounds (Connor, 1943) and that it is this combination which is responsible for the typical Cheddar flavour. If this were so, the determination of total concentration of H_2S in cheese would not be a direct measure of its contribution to Cheddar flavour.

The author is grateful to Drs W. A. McGillivray and P. S. Robertson for their advice and interest, and to Miss Hannah Watt for technical assistance.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 243

Biological estimation of oestrogenic activity in red clover (*Trifolium pratense*): relative potencies of parts of plant and changes with storage

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(*Received* 31 *December* 1962)

Summary. Immature ovariectomized mice were used to compare the oestrogenic activities of different parts of the same clover plants; to estimate the relative potencies of biochanin A, genistein and diethylstilboestrol; and to compare the effects of different methods of storage on the oestrogenic potency of red clover leaf and petiole. Test materials were incorporated in the diet fed to the mice and the uterine weight response was used to measure oestrogenic activity.

With material harvested at four different times, the leaf or leaf and petiole had the highest activity; small and large stem fractions of the plant were both relatively less active, and blossom and seed head were inactive.

Comparison of successive estimates of the relative potency of isoflavones and diethylstilboestrol indicated that the relative responsiveness to the two types of oestrogen did not remain constant. Thus comparisons of estimates of oestrogenic activity of plant material, obtained in terms of diethylstilboestrol in different experiments, could be invalid.

The potencies of alcohol, acetone and freeze-dried preparations were compared after storage for 18, 115, 212 and 230 days. Of the procedures tested, the most satisfactory was that in which fresh material was placed in 95% alcohol and the liquor and residue dried and stored at room temperature over concentrated sulphuric acid at reduced pressure.

When considering the possible effects of oestrogenic pasture plants on grazing animals, it is important to know the relative activities of the various parts of the plant at different stages of growth and at different times. With subterranean clover, Robinson (1949), Alexander & Watson (1951) and Bennetts & Underwood (1951) have shown that the potency of the leaf was higher than that of the petioles, stems or roots. Similarly, in ryegrass and cocksfoot as well as in red clover, Legg, Curnow & Simpson (1950) found oestrogenic activity to be greater in leaf than in stem. In oestrogenic samples of lucerne the highest activity was in the leaves (Pieterse & Andrews, 1956). In the present study the activity of parts of the red clover plant have been compared using materials harvested at four different times.

Where comparisons have to be made between samples of plant material collected at different times two approaches are possible. In the first, bioassays are carried out

immediately following harvesting, the comparisons being made between materials harvested at different times in terms of a reference oestrogen included as a standard treatment in each bioassay. Unfortunately, the isoflavone oestrogens, which would be the most suitable standards for estimations with red clover, are scarce and expensive and cannot be used as routine standards. Diethylstilboestrol and oestradiol have therefore been used (e.g. Cheng, Yoder, Story & Burroughs, 1954; Bickoff, Booth, Livingston, Hendrickson & Lyman, 1959). When the oral route of administration is employed diethylstilboestrol seems the best choice. It is readily available, cheap and usually gives a dose response line sufficiently parallel with those of oestrogenic plant materials or pure isoflavones (Munford & Flux, 1961; Wong & Flux, 1962). There is the possibility, however, that the relative sensitivity of test animals to two different types of oestrogen may differ from time to time. Evidence for this possibility is presented here.

In the second approach, with material collected at different times, all comparisons of interest are made in the same multiple bioassay. This avoids difficulties due to differential changes in sensitivity of the test animal, but introduces a further risk that potency may be lost during storage of plant material (Bickoff, Livingston, Booth, Hendrickson & Kohler, 1960; Flux, Munford & Barclay, 1961). The potencies of oestrogenic subterranean clover samples after treatment and storage in different ways were compared by Robinson (1949) who found that placing the fresh material in alcohol was superior to oven-drying. Alexander & Watson (1951) found that dehydration in ovens lowered the oestrogenic activity of subterranean clover as measured by bioassays, but little further change in activity occurred during storage over 17 months. In the present paper the effectiveness of several storage methods for red clover leaf and petiole have been examined.

MATERIALS AND METHODS

Animals

Ovariectomized mice, 21–23 days old, from the same colony as those employed by Munford & Flux (1961) and Wong & Flux (1962), were used as test animals. Diet, in which oestrogenic materials were incorporated, was fed at the rate of 2·5 g per mouse per day for 6 days. Six mice were used at each of two dose levels for every subsample of plant material tested. Body weights, uterine weights and the incidence of vaginal opening and positive vaginal smears were recorded.

Preparation of materials for bioassay

For the comparisons of the parts of the plant four plots of red clover were used, each being harvested at a different time. The plants taken from each plot were separated into leaf, petiole and large stem, so that each comparison was made between material from the same plants. Freeze-dried plant material was incorporated into the diet as described previously (Munford & Flux, 1961) at concentrations of 20 and 10% or of 10 and 5% dry plant material and the potencies estimated soon after harvesting. The plant material contents of the diets were equalized with non-oestrogenic ryegrass powder.

For the storage experiment the subsamples of leaf and petiole were prepared immediately after harvesting by the following methods:

(1) *Freeze drying*

(*a*) Frozen (in plastic bags at −15 °C); and (*b*) Desiccated (at reduced pressure over concentrated sulphuric acid at room temperature).

(2) *Alcohol treatment*

(*a*) Wet (left in alcohol until shortly before use and then prepared as in 2*b*); and (*b*) Desiccated (left in alcohol 1 to 2 weeks, liquor dried on to wholemeal flour in oven at 60 °C or in draught from electric fan heater; leaves dried, ground and mixed with material from liquor; stored at reduced pressure over concentrated sulphuric acid).

(3) *Acetone treatment*

(*a*) Wet (as for the alcoholic preparation); and (*b*) Desiccated (as for the alcoholic preparation).

Freeze-dried material was incorporated in the diet in the manner described previously (Munford & Flux, 1961). The subsamples treated with alcohol or acetone (300 g fresh material in 600 ml 95 % ethanol or 100 % acetone) were prepared for feeding by incorporating the mixture with wholemeal flour in the diet. The dose levels employed for all subsamples were obtained by including 5 and 10 % of the dried plant material in the diet. The content of plant material of the diets was equalized by adding dried non-oestrogenic ryegrass powder to the diets containing 5 % of dried clover.

Statistical methods

The methods used were the same as those described by Munford & Flux (1961).

RESULTS

Comparisons of oestrogenic potency have in this paper been made using the uterine weight response. The body weights of the mice on the different diets were similar. The incidence of vaginal opening and of positive vaginal smears added nothing to the information provided by uterine weights (cf. Munford & Flux, 1961) and these data are not presented.

Activities of different parts of red clover plants

In Table 1 the relative potencies of different parts of red clover plants are presented. The uterine weights were transformed to logarithms to remove a correlation between mean and standard deviation.

Blossom and dried seed head had little or no activity. The leaves had the highest activity, large or small stem and petiole were less active. Relative potencies of stem to leaf or leaf and petiole had very wide 95 % fiducial limits, but, in each instance where a comparison could be made, the stem was estimated to be about one-fifth to one-third as potent as the material containing leaf.

Comparison of different estimates of relative potencies of genistein, biochanin A and diethylstilboestrol

Munford & Flux (1961) and Wong & Flux (1962) have published relative potencies of genistein, biochanin A and diethylstilboestrol and these, together with some additional data of Wong and Flux, are presented in Table 2. These results suggest

Table 1. *Comparisons of oestrogenic potencies of different parts of red clover plants*

Part of plant or comparison	Dose, % of diet	Plot and harvest date: A 12. ii. 60	B 3. iii. 60	C 27. iv. 60	D 10. iii. 61
		Mean uterine weight of six mice, log mg			
Leaf	10	1·222	1·206	—	—
	5	1·006	1·093	—	—
Leaf and petiole	10	—	—	1·158	1·256
	5	—	—	0·967	1·012
Small stem and petiole	20	1·152	1·000	—	—
	10	0·946	0·830	—	—
Large stem	20	—	1·105	0·837	1·009
	10	—	0·968	0·849	0·908
Blossom	20	0·684	0·768	—	—
	10	—	0·780	—	—
Dry seed head	20	—	0·790	0·790	—
	10	—	0·762	0·804	—
Controls	0	0·756	0·760	0·778	0·769
Standard error of means		0·063	0·077	0·042	0·067
Least significant difference (5%)*		0·28	0·34	0·18	0·29
		Relative potencies and 95% fiducial limits			
Small stem and petiole to leaf		Not valid†	0·18 ($0·01 \times 10^{-8}$ to 0·38)	—	—
Large stem to leaf			0·34 ($0·05 \times 10^{-2}$ to 0·70)	0·26 ($0·06 \times 10^{-2}$ to 0·44)	—
Large stem to leaf and petiole			—	—	Not valid‡
			—	—	

* Calculated from the mean square in an analysis of variance, the number of items per mean (6) and the value of t ($P = 0·05$) for the number of degrees of freedom associated with the error mean square.
† Slope of the common regression line not significantly different from zero.
‡ Dose response lines not parallel, that for large stem was not significantly different from zero.

Table 2. *Comparisons of estimates of relative potencies of diethylstilboestrol and isoflavones*

	Relative potencies and 95% fiducial limits (diethylstilboestrol = 1)	Lambda*
Biochanin A		
(1) Wong & Flux (1962)	$9·1 \times 10^{-6}$ ($6·0$–$13·7 \times 10^{-6}$)	0·14
(2) Wong & Flux (unpublished)	$18·3 \times 10^{-6}$ ($12·9$–$26·6 \times 10^{-6}$)	0·15
Genistein		
(1) Munford & Flux (1961)	$8·9 \times 10^{-6}$ ($6·6$–$12·1 \times 10^{-6}$)	0·13
(2) Wong & Flux (1962)	$13·5 \times 10^{-6}$ ($10·3$–$17·3 \times 10^{-6}$)	0·14

* Defined by Emmens (1950).

that the relative responsiveness of the mice in the colony to diethylstilboestrol and to the oestrogenic isoflavones was not constant.

Comparison of the oestrogenic potencies of red clover leaf and petiole after preparation and storage by different methods

In Table 3 are represented the results of 4-point bioassay comparisons between subsamples of red clover harvested on 28 November 1960 and 7 December 1960. The freeze-dried subsample stored at −15 °C was not included in the first comparison. The indication of higher activity of the acetone-treated subsample of the sample harvested on 28 November 1960 was unexpected after only 18 days of storage. The residue remaining after evaporation of the same volume of acetone, from the same bottle as that used for the clover, gave no indication of oestrogenic activity when tested by the vaginal smear method in six primed ovariectomized mice (Emmens, 1950).

Table 3. *Comparisons of the oestrogenic potencies of red clover leaf and petiole subsamples prepared in different ways*

Storage	Preparation	Relative potency and 95 % fiducial limits
	Sample harvested 28. xi. 60	
Initial tests 18 days' storage	Freeze-dried and desiccated	1·0
	Alcohol and desiccated	1·14 (0·59, 2·88)
	Acetone and desiccated	2·16 (1·46, 2·08)
Intermediate tests 115 days' storage	Freeze-dried and desiccated	1·0
	Alcohol and desiccated	1·30 (0·77, 1·41)
	Freeze-dried and frozen	Assay not valid*
Final tests 212 days' storage	Freeze-dried and frozen	1·0
	Freeze-dried and desiccated	1·04 (0·91, 1·19)
	Alcohol and desiccated	1·53 (1·30, 1·81)
	Alcohol and wet	1·30 (1·08, 1·56)
	Acetone and desiccated	1·30 (1·18, 1·51)
	Acetone and wet	0·72 (0·62, 0·82)
	Sample harvested 7. xii. 60	
230 days' storage	Freeze-dried and frozen	1·0
	Freeze-dried and desiccated	0·88 (0·83, 0·94)
	Alcohol and desiccated	1·26 (1·00, 1·63)
	Alcohol and wet	1·32 (1·02, 1·71)
	Acetone and desiccated	1·40 (1·08, 1·82)
	Acetone and wet	1·56 (1·19, 2·04)

* Slope of dose response curve not significantly different from zero leading to departure from parallelism in the bioassay.

After 115 days' storage the freeze-dried and deep frozen subsample produced a less steep dose response line than those of the freeze-dried and desiccated, and alcohol-treated and desiccated subsamples which were very similar in potency. Because of a significant deviation from parallelism the freeze-dried and deep frozen samples could not be compared with the others by bioassay methods.

In the final comparison the two freeze-dried subsamples had very similar potencies, both less than those of the alcohol preparations. The acetone sample stored in a desiccator showed a similar potency to that of the alcoholic ones but the subsample stored in acetone showed an even lower potency than the freeze-dried ones.

The sample harvested on 7 December 1960 was tested at only one time, after 230 days' storage. The results were similar to those for the 28 November 1960 sample in that both alcoholic preparations had higher activity than the freeze-dried ones. The subsample stored in acetone, however, had an activity similar to that of the acetone subsample stored in a desiccator and to that of the alcoholic preparations.

DISCUSSION

The comparison of the relative potencies of the different parts of the plant requires little comment. As was found for subterranean clover (Robinson, 1949; Alexander & Watson, 1951; Bennetts & Underwood, 1951) the part of highest activity was the leaf. Stems had less activity per unit weight and apparently there was little or no activity in the flowerheads or seeds.

The comparison between the successive bioassay estimates of the oestrogenic activities of biochanin A and genistein suggested that the relative responsiveness of the mice to diethylstilboestrol and oestrogenic isoflavones changed with time. Mice of the same colony, prepared in the same way, were used on each occasion and no cause for the differences other than changing responsiveness could be suggested. With biochanin A one estimate was approximately double the other, a change sufficiently great to make diethylstilboestrol unsuitable as a standard for oestrogenic potency due to genistein and biochanin A except for very approximate estimates. If, as seems likely, all mice vary in relative responsiveness to different types of oestrogen, the range of relative potencies for diethylstilboestrol and the isoflavone oestrogens found in different laboratories [see discussion in previous papers, Munford & Flux, 1961; Wong & Flux, 1962] is not surprising.

The interpretation of the results of the storage experiment was made difficult by the variability of the comparisons and the lack of a standard preparation. Changing sensitivity to oestrogen with time in a mouse colony is not uncommon (Emmens, 1950) and this may have explained the results of Alexander & Watson (1951) who reported significant differences between responses to a 'standard' clover. But this would not account for the change in the present experiments where some dose response lines changed in slope or position relative to the others in successive bioassays.

Freeze-dried clover, stored frozen, gave a flat dose response line after 115 days' storage and a steeper one (not differing significantly in slope from the others) at 212 days. The acetone and desiccated material from the red clover sample did not change in relative slope, but appeared to lose about 50% of its potency relative to the other preparations, between the first and final tests. For the sample harvested on 28 November 1960 the acetone-treated preparations stored wet were less potent than the alcoholic preparations but this was not so for the corresponding preparations made from the sample harvested on 7 December 1960.

The cause of the differences between the responses to the freeze-dried deep-frozen, and the acetone-desiccated peparations in successive tests is not known. Possibly variation in the responsiveness of mice, greater than those observed within assays, was responsible. Lack of a standard preparation made it impossible to tell, from the bioassay results themselves, whether the freeze-dried samples lost potency or the alcoholic ones gained it. However, previous evidence, including an example in which

oestrogenic red clover completely lost its ability to increase uterine weight after a year, has shown that freeze-dried red clover preparations lose potency during storage (Flux *et al.* 1961). Alcohol would be expected to prevent enzymic activity leading to changes giving greater oestrogenic potency. Hence it seems probable that the differences in the final tests were caused by the alcoholic preparations maintaining their potencies better than did the freeze-dried ones.

On the basis of evidence obtained in these experiments it appears that if samples of red clover must be stored for long periods before testing them for oestrogenic potency, alcoholic preparations are likely to be more satisfactory than freeze-dried ones. This conclusion is in agreement with Robinson (1949) for subterranean clover with the proviso that in his work the comparison was between alcoholic preparations and oven-dried clover.

The authors are grateful to Dr E. Wong for permission to use unpublished results; to Mr T. S. Ch'ang for the provision of some of the red clover samples and to Messrs C. Fairless and E. Ormsby for technical assistance. Research grants to the senior author from the New Zealand Universities Grants Committee and the Department of Scientific and Industrial Research (N.Z.) are gratefully acknowledged.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 251

Inherited casein variants in cow's milk

II. Breed differences in the occurrence of β-casein variants

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(*Received* 15 *January* 1963)

Summary. A survey was made of the frequency of β-casein variants in the milk of cows of five major British breeds. Three variants A, B and C differing in electrophoretic mobility were found to occur either singly or in pairs, as expected if each were determined by a single allele. The A variant was the most common and found in all breeds. Ayrshires and Shorthorns produced no other variant, whilst the B variant occurred in Jerseys and, at low frequency, in Guernseys and Friesians. Production of the C variant was restricted to the Guernsey breed in which it occurred at low frequency. The technique of paper electrophoresis, used for β-casein 'typing' of some 600 cows, is described in detail.

The synthesis of β-casein in the mammary gland of the cow has been shown to be under the control of three alleles of a gene, *β-Cn*, capable of causing three electrophoretically distinguishable variants, A, B and C, to be formed either singly—in homozygotes—or paired—in heterozygotes (Aschaffenburg, 1961, paper I of the present series). Initial results suggested that the β-casein polymorphism might not be universal, but restricted to certain breeds of cattle and, accordingly, a survey was made of the β-caseins in the milk of some 600 cows belonging to five major British breeds. The results of this survey are reported here, together with a more detailed description of the technique used for 'typing' the animals than that given in the earlier communication. The survey was completed before it became known (Thompson, Kiddy, Pepper & Zittle, 1962) that another casein component, α_s-casein, also occurs in three variants. These can be differentiated by starch gel electrophoresis, but not by the technique of paper electrophoresis described below.

EXPERIMENTAL

Sources of milk samples

Milks from individual Jerseys, Guernseys, Ayrshires, Shorthorns and Friesians were examined for the occurrence of β-casein variants. For all breeds except the Friesian the basic testing arrangement was the same: small sets of milk samples, usually eight, were collected from several farms, rather than larger numbers of samples from a particular farm. This method avoided testing too many close relatives in a herd and gave a reasonable cross-section of the breed. Larger numbers of 'follow-

up' samples were, however, collected in a few instances in which a genetical picture of special interest emerged.

With the fifth breed, the Friesian, a different approach was used: in addition to the cows of the main Institute herd, two collections of heifers from herds in the South of England, usually two to four animals from a given farm, happened to be available at the Institute during the period of the survey. Their milk too offered a good cross-section of the breed.

Collection of milk samples

Samples of morning or evening milk were collected in 3-oz bottles containing one drop of formalin solution as a preservative. This mode of preservation does not interfere with β-casein typing, though it causes a slight blurring of the electrophoretic bands. No preservative was added when it was known that samples would reach the laboratory within a short time after milking.

Preparation of milk samples for paper electrophoresis

The caseins were prepared for electrophoresis by the following simple procedure. The milk is warmed to about 40 °C, centrifuged, and the skim-milk syphoned off. Ten ml of skim-milk is pipetted into a 50 ml centrifuge tube and diluted to approximately 40 ml by adding distilled water warmed to about 40 °C. The casein is precipitated by addition of 1 ml of a 1:10 dilution of glacial acetic acid, followed by 1 ml of 1 M-sodium acetate. After not less than 10 min the casein precipitate is spun down, the supernatant decanted, the precipitate washed by stirring it in warm water, and centrifuged again. The supernatant is poured away, 2 g of urea of reagent quality added to the wet casein and the precipitate dispersed to a slightly cloudy solution with careful addition of a little distilled water. Dispersion is aided by immersing the tube in a beaker containing warm water. The final volume is adjusted to approximately 5 ml, giving a solution with a casein content of the order of 5 %. If cold-stored, the solution must be warmed before it is used for paper electrophoresis.

Paper electrophoresis

Moving-boundary electrophoresis has contributed much to our present knowledge of the casein complex, but the method does not lend itself to serial comparisons of large numbers of samples, and it requires lengthy sample preparation in order to convert the opaque caseins into optically clear solutions. The advantages of electrophoresis on supporting media, such as filter paper, are thus self-evident. However, satisfactory resolution of the casein components by paper electrophoresis proved to be far from easy. Experiments on the lines suggested by Macrae & Baker (1958) and by Sode-Mogensen & Lahav (1960), working with conventional buffer systems, and by Zhdanova & Vlodavets (1959), working with buffered urea solutions, failed to provide a clear-cut separation of the major casein constituents. The incorporation of urea improved the results by providing less ragged bands, particularly of the leading α-casein component. Further experiments showed that the degree of resolution depended markedly not only on the pH and ionic strength of the chosen buffer, but also on the nature of the ions of which it is composed, and led to the selection of a citrate-phosphate buffer of the composition given below. It is noteworthy, and

possibly not fortuitous, that the constituents of this buffer are associated with the casein complex as found in milk. In its final form the paper electrophoretic technique permits resolution of the caseins into the clearly separated bands shown in Fig. 1, and in Figs. 1 and 2 of the earlier paper (Aschaffenburg, 1961). At the time of publication of the earlier paper the band seen in front of the β-caseins had not been characterized; its identity with κ-casein has since been established.

Technique. Several types of apparatus were tested: those in which the filter paper is arranged horizontally gave better results than those of the hanging strip type, and

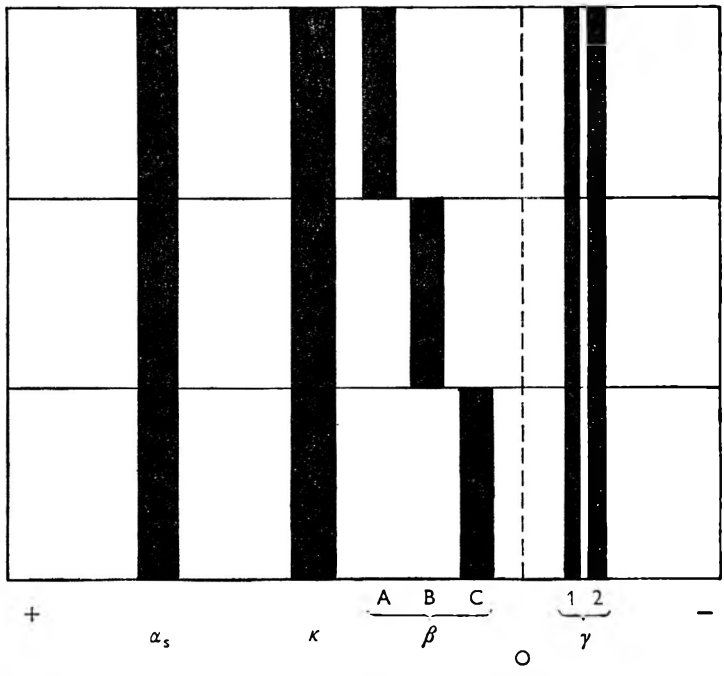

Fig. 1. Separation of casein components by paper electrophoresis. O = origin.

the compact unit described by Kohn (1957), available from the Shandon Scientific Company Ltd., 6 Cromwell Place, London S.W. 7, was used throughout the work. The adjustable end-bars on which the papers rest are set 14 cm apart. The apparatus is charged to a depth of 2 cm with a buffered urea solution made up of the following chemicals of reagent grade: 0·913 g of citric acid monohydrate, 3·56 g of disodium hydrogen phosphate, 300 g of urea, distilled water to 1 l. Originally 6 M-urea was used (Aschaffenburg, 1961), but the 5 M solution now recommended gives equally good results.

The apparatus will hold two sheets, 10 × 20 cm, cut from Whatman 3 MM filter paper of chromatography grade, permitting simultaneous electrophoresis of eight samples. To apply the samples each paper is drawn through the solution of buffered urea, placed on blotting paper for drainage of excess solution and then on a glass plate. The area of application is blotted further by gentle pressure from a strip of the filter paper approximately 1 cm in width. The samples are applied with a fine camel-hair brush across marked distances of 1·5 cm each. The best line of application for

β-casein typing is not the exact centre of the long axis of the paper, but one a few mm displaced towards the cathode. The precise position cannot be laid down, as it was found to vary from apparatus to apparatus, but it can easily be ascertained for each apparatus by comparative tests with the same sample applied to different application lines.

Preliminary trials showed that electrophoresis at a low temperature produced results superior to those obtainable at room temperature, and accordingly our runs were made in a cold-room at about 4 °C. The papers are placed into position, left for 30 min to allow equilibration of temperature and buffer flow, and a constant voltage of 180 V is then applied for 5½ h, producing an initial current of approximately 0·45 mA/cm which rises slightly as the run proceeds.

At the end of the run the papers are suspended horizontally and dried at about 105 °C for 15–20 min. They are then stained with bromophenol blue, using a tenfold dilution of a stock solution, prepared by dissolving first 5 g of bromophenol blue and then 100 g of mercuric chloride in 900 ml of methanol, and finally adding 200 ml of glacial acetic acid. After a staining period of about 3 min, the background stain is removed by washing several times in tapwater containing 1 % (v/v) glacial acetic acid. When the unbound dye is removed, after 30–40 min, the papers are suspended vertically and allowed to dry in the air. To develop the full colour of the protein-bound dye, it is advisable to expose the papers to ammonia vapour whilst they are still slightly damp.

RESULTS

Jerseys

Nine sets of eight milks each were examined with the following results.

	β-casein phenotype		
Herd no.	A/A	A/B	B/B
J 1	8	0	0
J 2	1	6	1
J 3	4	2	2
J 4	8	0	0
J 5	8	0	0
J 6	5	3	0
J 7	1	4	3
J 8	1	2	5
J 9	6	1	1
	42	18	12

These data provide conclusive proof of the existence of a β-casein polymorphism in Jersey cattle. The frequency with which the B-allele occurs varies remarkably from herd to herd, but overall it is fairly high. That it can reach very high proportions on a particular farm is illustrated by the findings for herd J 2 from which follow-up samples were collected. The distribution of phenotypes in a total of forty-three samples (including those listed above) was as follows.

A/A	A/B	B/B
9	22	12

An examination of family data showed that thirty-one of the forty-three animals

were the offspring of six bulls, certain (nos. 3, 4 and 5) or likely to transmit the B-allele.

Bull no.	No. of matings	Phenotype of offspring A/A	A/B	B/B
1	9	0	5	4
2	7	2	5	0
3	6	1	2	3
4	3	1	1	1
5	3	1	1	1
6	3	0	2	1

Guernseys

Twelve sets of eight milks each were examined. Variants of β-casein were found at only four of the twelve farms, whilst all the animals at the remaining eight farms produced the A variant alone.

Herd no.	β-casein phenotype A/A	A/B	B/B	A/C	B/C	C/C
G4	4	2	0	2	0	0
G7	6	2	0	0	0	0
G8	5	0	0	1	0	2
G12	5	0	0	3	0	0

Clearly the variants B and C occur in Guerney milks, but in very low frequency. Follow-up tests at three of the farms gave the following results (including those listed above).

Herd no.	No. of samples tested	β-casein phenotype A/A	A/B	B/B	A/C	B/C	C/C
G4	40	28	2	0	10	0	0
G7	16	12	3	1	0	0	0
G8	39	21	0	0	16	0	2

These results put the issue beyond doubt as far as the C-allele is concerned, but the rarity of the B-allele raised the question whether its occurrence might not be a reflexion of cross-breeding with Jersey cattle. Recourse was therefore had to an examination of milk samples from the island of Guernsey itself where the purity of the breed can hardly be regarded as suspect. Eight sets of six milks each were tested, revealing the occurrence of three of the six potential phenotypes.

Herd no.	β-casein phenotype A/A	A/B	A/C
IG 1	6	0	0
2	5	*1*	0
3	6	0	0
4	5	0	1
5	4	*1*	1
6	5	0	1
7	5	0	1
8	5	0	1

These findings allow the conclusion that the B-alleles is a genuine constituent of Guernsey milk. Its rarity is reflected in the fact that only one homozygote B/B, and

no heterozygote of type B/C was encountered in examining the milk of over 200 Guernsey cows. The high frequency of the C-allele in the animals tested in herd G.8, the only herd in which homozygotes for the C-allele were met with, can be explained by the breeding regime of this farm. No less than twenty-seven of the thirty-nine animals tested were found to be offspring of one particular bull (I) and of three of his sons (II, III, IV).

Bull no.	No. of matings	Phenotype of offspring		
		A/A	A/C	C/C
I	10	5	5	0
II	6	3	2	1
III	5	2	3	0
IV	6	4	2	0

There can be little doubt that the sons as well as the parent bull transmit the C-allele, though this is proven only for bull no. II which must be a heterozygote A/C.

Ayrshires and Shorthorns

For both breeds eight sets of eight milks each were examined. Every one of the sixty-four animals of each breed produced only one β-casein, the A-variant, a result confirming the suspected restriction of the β-casein polymorphism to certain breeds of cattle.

Friesians

The Institute cows. Of the seventy-five milk samples examined, seventy-three produced a single β-casein, the A-variant, one was classified as A/B, whilst the remaining one was difficult to classify. The single β-casein band obtained on paper electrophoresis had a mobility a little slower than A, but faster than B. The anomaly, referred to as β-casein A_{slow}, will be discussed later.

Heifer collection I. This group consisted of thirty-six animals of which thirty-three were typed as A/A, one as B/B and one as A/B; the remaining cow provided another example of A_{slow}. Ten contemporaries of the non-pedigree B/B-animal were sampled at the farm of origin: all of them were found to be A/A. The cows classified as A/B and A_{slow} were two of a group of four from another non-pedigree herd. Of twenty-five follow-up samples taken at the farm of origin, twenty were A/A and five A/B.

Heifer collection II. This group consisted of forty-six animals of which thirty-nine were typed as A/A, and seven as A/B. The A/B-animals came from six different herds, four of which were of pedigree stock. Follow-up samples were obtained from all the pedigree, and one of the non-pedigree herds.

Herd no.	No. of samples	β-casein phenotype		
		A/A	A/B	B/B
1	12	11	1	0
2	16	14	1	1
3	16	14	2	0
4	16	16*	0	0
5 (non-pedigree)	16	14	2	0

* One of the cows was of the A_{slow} type.

In view of the low frequency of the B-allele it is not surprising that family studies were of little reward. A few pointers were obtained, e.g. that the dam of one of the A/B-animals housed at the Institute was of the same A/B type, and that the A/B and B/B-animals in herd no. 2 belonged to the same breeding line as the A/B-cow from this farm kept at the Institute.

Taken together, the results of the survey of β-caseins in Friesian cattle suggest strongly that the B-allele is a true constituent of the caseins of this breed rather than a reflexion of cross-breeding with Channel Island cattle, as was at first suspected.

DISCUSSION

This survey of the milk of cows of five major British breeds has shown that the β-casein polymorphism is not universal. Only one variant, A/A, was found in Ayrshire and Shorthorn milks, whilst the remaining breeds, Jersey, Guernsey and Friesian, had a second variant, B, in common. A third variant, C, was found in addition only in the milk of Guernsey cows. The establishment of a true genetic difference between the two Channel Island breeds is perhaps the most significant finding of this work. It is an open question whether the C-allele in Guernsey milk is of comparatively recent or of ancient origin, in which latter case it would point to differences in the derivation of Guernsey and Jersey cattle. A search for the C-allele in European, and particularly French, breeds is highly desirable, as it might throw light on the pathway by which cattle reached the Channel Islands. It is noteworthy that a study of haemoglobin polymorphism would be of no help in this respect, as both Channel Island breeds are characterized by the production of the same two haemoglobin variants (Bangham, 1957).

Another difference between the two Channel Island breeds is seen in the frequency of occurrence of the B-allele, high in the Jersey, but low in the Guernsey breed. The presence of this allele in some, though few, Friesian milks is intriguing, as it suggests a possible link with the Channel Island breeds.

As a result of the work reported here, it is now possible to isolate the three β-casein variants from the milk of the appropriate homozygotes, and to establish the differences in their amino acid composition, the existence of which is clearly indicated by the variations in electrophoretic mobilities. It might also be possible, in due course, to locate the positions on the amino acid chain at which amino acid interchanges occur. Such studies should help in arriving at a better understanding of the structural features and chemical reactivity of the β-casein molecules.

As mentioned above, three instances were encountered in which Friesian cows produced the β-casein referred to as A_{slow}. Two of the cows are still at the Institute, and their milk has recently been re-examined after they calved down again, and the anomaly was found to persist. When the samples were studied by a more searching electrophoretic technique, now under development, which is based on the use of acrylamide gel as the supporting medium, the β-caseins resolved, surprisingly, into two bands indistinguishable from those normally obtained with milk samples from cows heterozygous for A and B. It is, however, evident from the anomalous behaviour of the A_{slow} samples on filter paper, that they cannot be classified as straightforward heterozygotes A/B, and they require further investigation.

As recently shown by Thompson *et al.* (1962), α_s-casein as well as β-casein occurs in the form of three variants in cow's milk. Attempts to resolve the α_s-variants by paper electrophoresis have so far failed. Future surveys of the kind described here will, therefore, require the application of other techniques, e.g. of starch gel electrophoresis, as used by the American authors, or of the above-mentioned acrylamide gel method which promises to be simpler to use. Nevertheless, a fairly detailed description of the technique of paper electrophoresis was felt to be justified, as the simple method retains its usefulness in other respects. It remains, in particular, the only means by which the elusive κ-casein component can be isolated in the form of a single well-defined band.

The prediction that several independent genes are involved in the elaboration of the casein complex by the mammary gland (Aschaffenburg, 1961) has been borne out by the discovery of the α_s-casein polymorphism by Thompson *et al.* (1962), and with the searching methods now available, similar findings concerning some of the lesser constituents of the casein complex may well emerge before long. Whatever the outcome of future investigations, it is already clear that not one, but a series of genes is involved in the synthesis of the unique phosphoproteins which form the principal source of protein nutrition of the mammalian young.

It is a pleasure to repeat my thanks to my colleagues of the Dairy Husbandry Department, Dr F. H. Dodd and Mr W. S. Mitchell, and to Mr N. T. Culling and his staff at the Reading Regional Milk Records Office, Milk Marketing Board, for the collection of samples and information. I am also grateful to Mr E. Baker, Guernsey, for arranging the collection and dispatch of milk samples from the island. Last, but not least, I thank Miss J. E. Cooper who assisted with skill and enthusiasm.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 259

Preparation of β-casein by a modified urea fractionation method

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(*Received* 15 *January* 1963)

It has been shown by Hipp, Groves, Custer & McMeekin (1952) that the differential solubility in urea of the casein components of cow's milk can be utilized for preparative purposes. Fractionation is achieved by step-wise dilution of a solution of all the casein components in strong (6·6 molar) urea. In connexion with the work on the three genetically distinct β-caseins discussed in the preceding paper, the need arose to prepare the variants A, B and C from the milk of appropriate homozygotes. This led to the development of the modified procedure described in this note, a procedure more sparing in urea and more effective in the initial isolation of the β-casein fraction than the original method of Hipp *et al.*

Principle of the method. The total casein obtained by acid precipitation is dispersed in 3·3 M-urea, and the pH reduced to 4·6. Under these conditions the bulk of the β-casein remains in solution whilst virtually all the other casein components are precipitated, as illustrated in Pl. 1. The β-casein can now be purified by conventional methods.

Method. The casein is precipitated from 1 l of skim-milk with N-HCl at pH 4·6, the precipitate filtered through muslin, washed with warm water, re-dispersed with the aid of N-NaOH, and precipitated again at pH 4·6. The filtered precipitate is now dispersed in about 700 ml of water by adding 180 g of urea of reagent quality and N-NaOH to a final pH of 7·5. The volume is adjusted to 900 ml, and the pH reduced to 4·6. The components insoluble in 3·3 M-urea settle in the form of oily floccules.

The supernatant, containing the β-casein, is filtered through Whatman No. 4 paper, the pH of the cloudy filtrate (about 800 ml) adjusted to 4·90, the filtrate diluted with 2 l of water and warmed to 30 °C. The flocculant precipitate is allowed to settle overnight. As much as possible of the supernatant is decanted, and the precipitate collected on a Buchner funnel.

The crude β-casein is dispersed in 400 ml of 3·3 M-urea with addition of NaOH to pH 7·5, and the turbid solution is brought to pH 4·60 and then warmed to 37 °C. The small oily precipitate is centrifuged down, and the supernatant decanted and filtered through Whatman No. 30 paper. The filtrate is brought to pH 4·90, diluted with 800 ml of water and warmed to 30 °C. The oily floccules of β-casein are centrifuged down, and further purified by dispersion in 300 ml of water with addition of NaOH. The β-casein is then re-precipitated at pH 4·90. The gummy precipitate is washed in succession with water, absolute alcohol and acetone, pre-dried in air, crushed to a fine powder, and finally dried *in vacuo*.

The yield of purified β-casein is usually of the order of 3 g/l, but it can be improved by increasing the volume of skim-milk used as the starting material. Paper electro-

phoresis shows the β-casein preparation to be free from major impurities. In particular no traces of α_s- and κ-casein can be detected.

The technical assistance of Miss J. E. Cooper is gratefully acknowledged.

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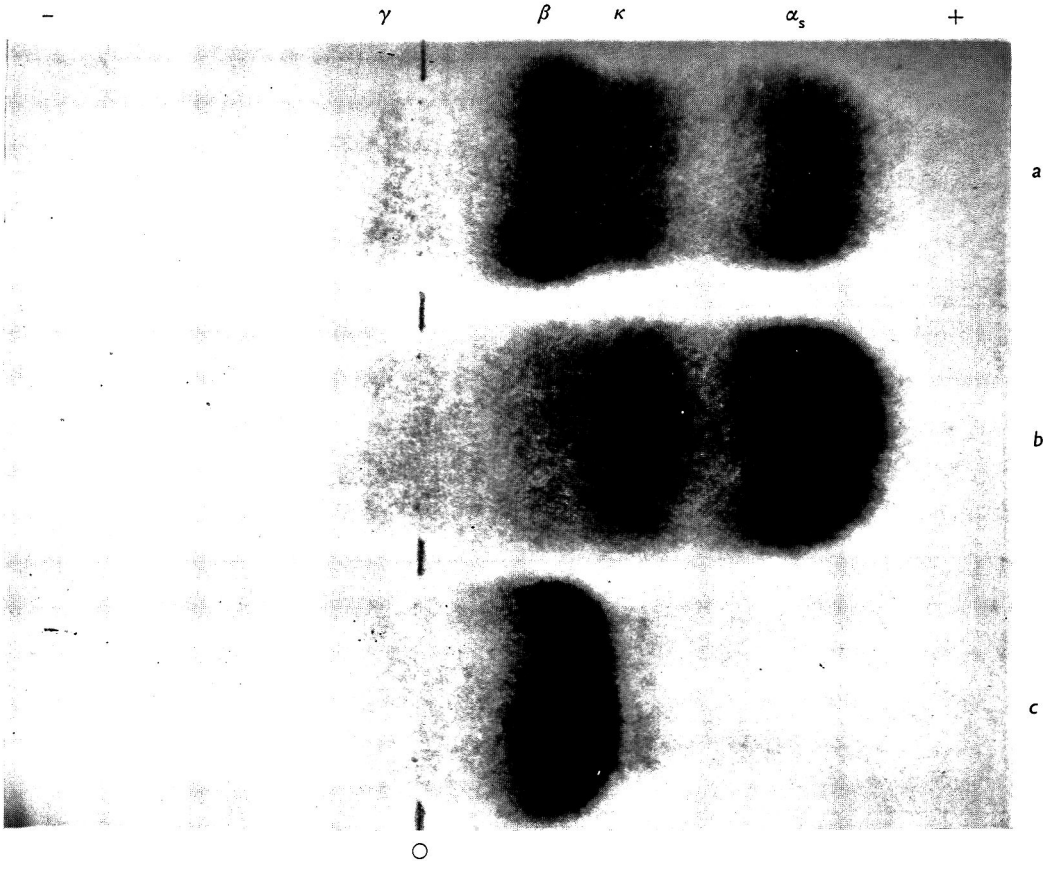
EXPLANATION OF PLATE

Plate 1. Fractionation of casein. *a*, total casein; *b*, components insoluble in 3·3 M-urea at pH 4·6; *c*, β-casein which is soluble under these conditions. Paper electrophoresis in 5 M-urea, buffered with citrate-phosphate, pH 7·15. ○ = origin.

Printed in Great Britain



(*Facing p.* 260)

Reviews of the progress of dairy science

Section A. Physiology

Part 1. The physiology of the rumen

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Part 2. Rumen microbiology

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(*Received* 4 *February* 1963)

CONTENTS

Part 1

Part 2

The physiology and the microbiology of the rumen are treated with such independence in the literature that it is difficult to survey them as a single subject, however closely related they may be inside the rumen. The subjects are therefore considered separately in the two parts of this review. These are linked by cross-reference where appropriate and are followed by a joint list of references.

Part 1. THE PHYSIOLOGY OF THE RUMEN

INTRODUCTION

The physiology of the rumen has attracted so much attention that it no longer seems possible to review it critically, readably and within a reasonable space and at the same time mention all the relevant work that has been published. The number of

references given has therefore been reduced by citing only the most pertinent or most recent from groups of papers, through which the others may be traced, and attention has been limited largely to the last 5 years or so by referring freely to books and reviews.

Rumen physiology was last reviewed in this *Journal* in an article by Edwards (1955) on 'The biochemistry and microbiology of the rumen'. Part 1 of the present review considers the rumen from an angle that highlights the physiological mechanisms by which conditions in the rumen are kept suitable for continuous microbial activity and growth. As a result of this approach some important aspects of rumen physiology, such as rumination and rumen movements generally, are not treated separately but are only touched on in passing. For the sake of brevity the reticulo-rumen will usually be referred to as the rumen.

The volume and composition of the rumen contents depends on the balance achieved between gains and losses, between the addition of food, water, salivary secretions and microbial products, and the removal of material by onward flow to the omasum, belching, absorption through the rumen wall and microbial degradation. The equilibrium that results is unstable to the extent that it varies with the diet and the feeding cycle and it may collapse under such extreme conditions as are imposed by diets containing excessive amounts of readily digested carbohydrates or urea. Normally, however, the rumen contents supply the basic requirements of the micro-organisms and conditions are constant enough for continuous if rather uneven microbial activity and growth. End-products of microbial metabolism are removed so that they do not eventually arrest further fermentation as they do in the silage heap, the brewer's vat and in some 'artificial rumens'. Functions such as salivary secretion, rumination, belching and reticulum and rumen contractions are largely controlled by physiological reflex mechanisms responding to stimulation of the mouth, reticulum, rumen and abomasum, and normally they may well depend on the summation of both excitatory and inhibitory stimuli.

ANATOMICAL AND PHYSIOLOGICAL DEVELOPMENT OF THE RUMEN

The rumen of the adult sheep is entirely lined by keratinized squamous epithelium. The histological appearance of the epithelium has been described by Dobson, Brown, Dobson & Phillipson (1956) and Lindhé & Sperber (1959) have studied the tissue by electron microscopy. The surface area of the epithelium is greatly increased by being thrown up into numerous folds and papillae which are supplied by a profuse capillary network. This large surface area, together with the columnar arrangement of mitochondria in the basal cells of the epithelium (Dobson *et al.* 1956) and the wide intercellular spaces (Lindhé & Sperber, 1959), probably accounts for the rapid passage of some solutes through the epithelium in both directions and for active absorption of sodium ions. No glandular cells are present in the reticulum or rumen of sheep and cattle and no digestive juice is secreted; the salivary glands supply virtually the only secretion entering the rumen but supply it in abundance. In the camel, on the other hand, peculiar sacculations of the rumen and most of the reticulum and omasum are lined by a glandular epithelium (Hansen & Schmidt-Nielsen, 1957) and another mammal that pre-ferments its food, the quokka, described by Moir, Somers & Waring (1956), does so in a glandular fore-part of the stomach.

The fore-stomach is small and almost empty at birth; suckling and swallowing of

fluid cause reflex closure of the oesophagal groove (Comline & Titchen, 1961) so that milk by-passes the rumen and enters the abomasum directly. The fore-stomach grows rapidly in animals at pasture but is arrested at an early stage of development when the diet is restricted to milk (Tamate, 1956). Calves and kids begin to ruminate when about 2 weeks old provided that roughage is available as fodder (Swanson & Harris, 1958; Tulbaev, 1958). Wardrop & Coombe (1960) measured the weights of the stomach compartments at weekly intervals in lambs at pasture with their dams and found that the rumen grew fastest, followed by the reticulum, the omasum and the abomasum and that these compartments reached their adult proportions by about 56 days. Church, Jessup & Bogart (1962) obtained similar results. In kids the capacity of the rumen increases from about $\frac{1}{2}$ to 3 l within 5 weeks of weaning (Tamate, 1957) but the actual volume of rumen contents increases more slowly (Wardrop & Coombe, 1961). The consumption of wood shavings by calves was found to increase the volume of fluid in the rumen and its rate of flow to the omasum (Smith, 1961) while the introduction of plastic sponges into the rumen increased both its capacity and musculature (Tamate, McGilliard, Jacobson & Getty, 1962).

Neither of the last two procedures encouraged papillary growth, which occurs quite independently of increases in the volume of the rumen and evidently depends on the establishment of an actively fermenting microbial population (see p. 289). Papillary growth is most rapid when rapidly fermented diets are fed, is slower on hay or grass, and fails altogether when only milk is given (Brownlee, 1956; Warner, Flatt & Loosli, 1956). Flatt, Warner & Loosli (1958) found that a synthetic diet, or the administration of a mixture of fatty acids, promoted papillary growth and a later paper (Sander, Warner, Harrison & Loosli, 1959) reported that the administration of sodium butyrate or sodium propionate into the rumen was sufficient to cause good papillary development, perhaps as a result of the metabolic reactions they stimulated in the rumen epithelium. Tamate *et al.* (1962) found that although the papillae are short at birth they shorten still further in calves fed only milk, perhaps as a result of the removal of the influence of maternal volatile fatty acids. Carbonated water or sodium and potassium chloride or bicarbonate caused little papillary growth; milk did cause some growth but only when given into the rumen. The histological development of the papillae is complete by about 40 days of age in grazing lambs (Church *et al.* 1962). Keratinization is increased by pelleting the ration when hay and grain is fed (Thompson, Kintner & Pfander, 1958).

Wardrop & Coombe (1961) found that the development of rumen function in lambs at pasture was characterized by a transition phase between non-ruminant and adult stages which extended from 3 to 8 weeks of age. During this phase the concentration of volatile fatty acids rose and that of ammonia fell irregularly while the pH fluctuated considerably. Early weaned calves also pass through a period during which rumen pH values may drop below 5 and the animals suffer from bloat and scouring (Dinda, 1960). Despite this evidence of rumen dysfunction young lambs digest lucerne chaff (Wardrop & Coombe, 1961) and 3- to 10-week-old calves digest grass (Preston, Archibald & Tinkler, 1957) quite efficiently.

Both the ability to absorb fatty acids from the rumen and salivary secretion only begin to develop in the very young ruminant with the onset of rapid fermentation in the rumen. This may explain why the pH and fatty acid concentrations are so

unstable when solids are first eaten. The rate of absorption of acetic acid increased with age in calves that were fed milk, alfalfa nuts and grain but remained at a low level when only milk was given (Sutton, McGilliard & Jacobson, 1961). Salivary secretion develops along similar lines. In confirmation of the observations of Elovskikh (1937*a*), Kay (1958) found that the parotid glands of calves less than 3 months old secreted little saliva. Subsequently the rate of parotid secretion was measured under anaesthesia in young goats that had been reared on milk and hay (Kay, 1960*b*). The glands secreted little saliva and appeared histologically immature during the first 2 weeks of life, but the rate of secretion per gram of parotid gland then increased until adult rates of secretion and histological appearance were achieved at about 3 months. The composition of the saliva showed little change with age. Wilson & Tribe (1961) showed that the growth and the physiological and histological maturation of the parotid glands were closely linked with the growth of the rumen and the establishment of ruminal fermentation. Development of parotid and rumen function was fastest in lambs at pasture, slower in lambs that were fed hay and almost completely arrested in lambs fed only milk. On the other hand, the submaxillary glands grew independently of the diet. The nature of the link between rumen and parotid development is an interesting problem which has still to be solved.

ORGANIC MATTER IN THE RUMEN AND FOOD INTAKE

The amount of organic matter in the rumen and its quality and concentration are clearly the most important factors that normally influence and limit microbial activity (see p. 292). The physiological regulation of the organic matter content of the rumen is discussed in recent reviews by Corbett (1961) and by Balch & Campling (1962) on feeding and the control of food intake, so most aspects of the subject may be dealt with rather briefly here.

Analyses of the contents of the various compartments of the gut in slaughtered animals, reviewed by Balch & Campling (1962), show that the rumen holds about three-quarters of all the dry matter contained by the digestive tract and that at least 60 % of the digestible dry matter of roughage diets is digested there. Similar results were obtained by Rogerson (1958). The importance of digestion in the rumen has also been demonstrated in conscious animals by direct measurement of the flow and composition of digesta leaving the omasum (Kameoka & Morimoto, 1959; Oyaert & Bouckaert, 1961) and entering the duodenum (Hogan & Phillipson, 1960; Harris & Phillipson, 1962; Ridges & Singleton, 1962). Boyne, Campbell, Davidson & Cuthbertson (1956) showed that the dry matter in the rumens of sheep on a diet of hay, ground maize and meals formed 75 % of the total dry matter in the gut when they were slaughtered immediately after feeding, but as a result of the decline in the weight and percentage of dry matter in the rumen contents between meals (Balch, 1958; Stewart, Stewart & Schultz, 1958) this figure fell to 60 % by 12 h after feeding. Balch & Line (1957) showed that the fall in body weight that occurs when cattle on predominantly hay diets are first turned out to pasture is largely due to a substantial decrease in the weight of the rumen contents.

The concentration of dry matter in the rumens of sheep is around 10 % on hay diets (as found by Gray, Pilgrim & Weller (1954), for example), is higher when con-

centrates are fed, and approaches 20 % on diets supplemented with maize (Boyne *et al.* 1956) or wholly of maize (Rogerson, 1958). It depends to some extent on the ash intake (Nicholson, Loosli, & Warner, 1960). Large fluctuations of the ratio of solids to water in the rumen are not to be expected for on most diets, maize being a significant exception, the volumes of water drunk and of saliva secreted are both roughly proportional to the dry weight of food eaten (see pp. 271, 275).

In most of these experiments the whole of the rumen contents was removed for measurement either by slaughtering the animal or by emptying the rumen completely through a fistula. This avoided the problem of having to obtain a fair sample of the rumen contents through a fistula, which is especially difficult on heterogeneous diets, such as hay and grain, which lead to stratification of many components of the rumen contents (Smith, Sweeney, Rooney, King & Moore, 1956). Rapid and even mixing of the rumen contents has been achieved in sheep by Sutherland, Ellis, Reid & Murray (1962) who used a pump to circulate the contents continuously through an external loop. It was necessary to feed grass nuts to avoid clogging the pump.

Control of food intake

The feeding value of a forage, as Crampton (1957) has remarked, depends more on the amount eaten than on its chemical composition. The voluntary intake of food by animals with simple stomachs generally falls as the digestibility of the diet increases; this ensures a reasonably constant intake of digestible energy. In ruminants the reverse is true; voluntary intake rises as the digestibility of the diet increases, at least with roughages (Blaxter 1950, p. 7). Consequently a small improvement in the digestibility of a fodder causes a large increase in the intake of digestible energy, for not only is the food more fully digested but also more is eaten. The voluntary intake of a forage is thus a good indication of its feeding value, and Crampton, Donefer & Lloyd (1960) have used the voluntary intake and digestibility of forage to compute a Nutritive Value Index.

The difference in the regulation of food intake between ruminants and non-herbivores seems to be related to the relative bulk and indigestibility of the ruminant's diet. The ruminant's gut is always partly filled with undigested food residues and it has long been thought that this might place a limit on the voluntary intake of roughages. The recent experiments of Blaxter and of Balch and their colleagues have convincingly shown that this is the case, and it follows that the amount of food that can be eaten depends on the rate at which residues from previous meals can be removed from the gut by digestion and by excretion in the faeces. Blaxter, Graham & Wainman (1956) measured the rate of passage of food through the sheep's gut and showed that grinding dried grass or increasing the amount eaten increased its rate of passage and so reduced its digestibility (see also Rodrigue & Allen, 1960). Later Blaxter, Wainman & Wilson (1961) measured the voluntary intake of poor, moderate and good hay and found that intake increased with the digestibility and rate of passage in such a way that the amount of dry matter contained by the gut at the end of a meal was the same, whatever the quality of the hay eaten. Similar results were obtained with steers (Blaxter & Wilson, 1962).

Balch and his colleagues stress the importance of the rumen. Campling & Balch (1961) found that the amount of hay eaten at a meal by cattle was increased by

removing swallowed food or rumen contents and was decreased by inflating a bladder in the rumen but not by pouring water into the rumen. The latter results have also been obtained with sheep by Lloyd Davies (1962). The intake of straw was less than that of hay and this was associated with slower digestion of fibre in the rumen and slower passage of residues through the gut (Campling, Freer & Balch, 1961). The straw diet was chewed and ruminated for much longer, per pound of food eaten, than the hay diet and this seemed to be needed to reduce the straw particles to a size that would pass through the reticulo-omasal orifice (Freer, Campling & Balch, 1962). Infusion of urea into the rumen, which accelerated both the digestion and rate of passage of straw, also increased the voluntary intake (Campling, Freer & Balch, 1962). Satiation did not depend simply on the amount of dry matter in the rumen at the completion of a meal since this was 35 % greater on hay than on straw although this difference narrowed to 6 % immediately before the next meal (Campling *et al.* 1961).

The evidence is thus strong that some stimulus associated with the bulk of food in the gut causes satiation but its exact nature and the location of the receptors sensitive to it need to be defined. Satiation and feeding centres are found in the goat's hypothalamus (Larsson, 1954; Wyrwicka & Dobrzecka, 1960) and presumably distension of the rumen stimulates the satiation centre just as stretch of the stomach does in other species (Sharma, Anand, Dua & Singh, 1961). Receptors sensitive to distension of the rumen may respond to changes in the volume of the rumen, to changes in tension in its muscular wall or both. Simple stretch of the rumen by the volume of its contents is unlikely to be the only important stimulus to satiation for Hydén (1961*b*) found little or no reduction in the volume of rumen fluid in sheep that were fasted for up to 5 days although certainly they must have been very hungry. If tension receptors in series with the rumen muscle are concerned their response may be modified by the increased muscular activity of the rumen during feeding and possibly by 'receptive relaxation' of the organ. Relaxation can be seen when the rumen is inflated by stages with gas, for the pressure falls off considerably between each step of the inflation (Kay & Phillipson, 1959).

The receptors may be similar to those located by Iggo (1955) near to the oesophageal groove. These appeared to be tension receptors in series with the muscle and accommodated slowly, if at all, to maintained stimuli. Hill (1959) has described nervous structures that form a network under the epithelium of the reticulum and rumen, especially in the regions that are most sensitive to mechanical stimulation. Touch and stretch of the cardiac and reticulo-omasal orifices and reticulo-rumen fold elicit different responses so there must be at least two sorts of receptors in these parts (Ash & Kay, 1959). Distension of the rumen by inflation or by balloons inhibits both rumination and salivary secretion (Elovskikh, 1937*b*; Kay & Philipson, 1959; Ash & Kay, 1959) and Wilson (1963*b*) has evidence that the inhibition of salivation observed at the end of a meal (Bailey & Balch 1961*a*, *b*) is due to the increase in the volume of rumen contents. This effect thus seems to resemble the satiation mechanism in some respects.

Distension of the lower gut has sharp inhibitory effects and also influences food intake. Distension of the abomasum inhibits rumen and reticulum contractions (Titchen, 1960), and omasal outflow (Ash, 1962*a*), while distension of the duodenum

inhibits abomasal outflow (Hogan & Phillipson, 1960) and abomasal secretion (Ash, 1961). Total obstruction of the flow into the duodenum (Ash, 1962*b*) or through the terminal ileum (Goodall & Kay, unpublished observations) rapidly leads to a complete loss of appetite. The importance of these inhibitory mechanisms in the regulation of food intake under normal conditions is unknown.

It is not certain what part the accumulation of food residues in the gut plays in limiting the intake of rich and digestible diets. Blaxter *et al.* (1961) found that a supplement of concentrates reduced the intake of poor quality hay less than that of good quality hay, suggesting that equality of distension may be the appetite-limiting factor in this situation. Balch & Campling (1962) suggested that the control of the intake of concentrated diets by ruminants may resemble that in non-herbivorous animals in being dependent on a variety of factors, including blood nutrient levels and heat production, in addition to gut distension. The concentrations of volatile fatty acids in the rumen increase too slowly after a meal for this to be a likely stimulus to satiation, and anyhow they are produced in greater quantity from the more digestible and therefore more readily eaten fodders.

Small animals need to consume more digestible energy per kilogram of body weight than large animals. Hungate, Phillips, McGregor, Hungate & Buechner (1959) studied a variety of ruminants in East Africa and found that the fermentation rate of the rumen contents was fastest in the smallest animal (a suni weighing 3·7 kg) but that the ratio of the weight of rumen contents to body weight did not vary with the size of the animal. This suggests that the high energy intake needed by the suni is achieved by rapid digestion and passage of food through the rumen rather than by increased capacity of the rumen. Such a small browsing animal may also be able to select more digestible food to eat. Zebus have a similar advantage over European cattle (Phillips, Hungate, MacGregor & Hungate, 1960; Phillips, 1961*a*).

Outflow from the rumen

The rate of flow of digesta from the rumen influences not only the food intake but also the volume and turnover of rumen fluid. Only the smaller and more fully digested particles of organic matter pass through the reticulo-omasal orifice (Weller, Pilgrim & Gray, 1962). Micro-organisms are swept along with the digesta so that the rate of flow through the reticulo-omasal orifice plays an important part in regulating their numbers. Those eaten by chance with the food but unable to multiply rapidly in the rumen are most affected (see p. 293).

The factors that influence the retention of particles in the rumen have been reviewed by Balch (1961) and by Balch & Campling (1962). These include the rate of digestion and the effect of urea infusions, the amount fed, the effect of grinding the food and the reduction of particle size by chewing during feeding and rumination, the specific gravity of the particles, and the movements of the rumen, reticulum and reticulo-omasal orifice. These will not be considered further here.

The outflow of fluid from the rumen has been reviewed by Hydén (1961*a*) who used polyethylene glycol, which is soluble, indigestible and unabsorbed, as a reference substance. The theory of this technique is examined more fully in a later paper (Hydén, 1961*b*). In sheep of about 50 kg, receiving hay or hay, beet and concentrates *ad libitum*, the volume of fluid in the rumen averaged about 4·5 l but was quite

variable. The outflow was about 300 ml/h, or 7·2 l/24 h, corresponding to 6% of the rumen contents hourly. In fasting sheep the flow fell after 2–3 days to about one-third of its initial rate although there was little change in the volume of fluid in the rumen. This is rather unexpected since the rumen contents become much more fluid during fasting. The flow rates agree reasonably well with earlier estimates on sheep (Bullen, Scarisbrick & Maddock, 1953; McDonald, 1958). Murray, Reid & Sutherland (1962) found the rumen outflow of sheep weighing 55 kg was about 6 kg/24 h when 1 kg of food was given as two meals daily, but was much less when the food was given continuously over the 24 h. The volume of rumen fluid in a 530 kg cow fed on hay was about 50 l and the outflow 150–170 l/24 h, a surprisingly high rate (Hydén, 1961*a*).

It has not yet proved possible to collect the material flowing through the reticulo-omasal orifice but direct measurements have been made on the omasal outflow. The absorption of water by the omasum (Gray *et al.* 1954; Oyaert & Bouckaert, 1961) will cause the omasal outflow to be less than the ruminal outflow, but the pattern of flow will probably be much the same since the omasum has little holding capacity. Oyaert & Bouckaert (1961) used a funnel placed in the proximal abomasum and fitted with a side-arm through which the omasal outflow could be diverted to the exterior at will. They report average hourly flows of 442–810 ml in sheep. These seem rather high compared with measurements of outflow from the rumen and it is probable that the flow was excessive due to failure to return the collected material to the abomasum. Ash (1962*a*) measured the flow through abomasal re-entrant cannulae placed immediately beneath the omaso-abomasal orifice, returning the digesta to the distal cannula frequently and in small volumes. The flow was greatly increased by adding 2·5–4 l of rumen contents to the rumen and was usually increased by feeding. It was doubled by failure to pour the collected digesta back into the abomasum and was reduced when extra material was poured in.

Frequency of feeding

Rats that are fed at intervals of 2 or 3 days resemble wild rats in that their gut is larger than that of frequently fed rats, allowing them to eat more at a meal, and enzymic activity and tissue oxidation rates are higher, enabling them to deal with the heavy if intermittent supplies of nutrients (Fábry, Petrásek, Kujalová & Holečková, 1962). Such adaptations, however, mean that food is not utilized as efficiently as when it is given as frequent small meals. In the case of protein, for example, frequent feeding allows a more even rate of absorption of amino acids which ensures that less amino acid is deaminated and oxidized or stored as fat.

One would not expect frequent feeding to be as important to ruminants, since slow fermentation of organic matter held in the rumen reservoir frees nutrients for absorption continuously between meals. Nevertheless, most experiments, the negative results of Rhodes & Woods (1962) being an exception, show that sheep and cattle benefit substantially. Young animals especially show improved weight gains, nitrogen retention, and decreased heat losses when a standard ration is divided into many meals instead of being fed all at once (e.g. Rakes, Lister & Reid, 1961). Sutherland (1962, pers. comm.) has found that the volume, composition and onward flow of rumen contents fluctuate very much less when sheep are fed slowly and continuously instead of twice daily. The main advantage of frequent feeding seems to be that fatty

acids are produced and absorbed more steadily so that less are oxidized and wastefully dissipated as heat.

A still clearer example of the disadvantages of over-rapid fermentation is provided by the fate of urea. If a urea supplement is given into the rumen as a drench ammonia is swiftly evolved with toxic consequences (see p. 280) and most of the nitrogen is excreted in the urine. If it is given mixed with food, or by continuous infusion, ammonia is released more slowly and more is incorporated into microbial protein and so made usefully available to the animal (Reid, 1953; Coombe, Tribe & Morrison, 1960; Campling *et al.* 1962).

The feeding routine adopted by ruminants at pasture may also influence how efficiently they utilize their food. Diurnal variations in microbial activity are considered in Part 2, p. 292.

Food selection

The quality of the food consumed by grazing animals depends on what they choose to eat as well as on the nature of the pasture (see Arnold, 1960, for example). Many wild ruminants in North America prefer browsing on trees and shrubs to grazing grasses and herbs (Martin, Zim & Nelson, 1951) and this serves as a useful reminder that pasture plants form a rather artificial diet for domesticated ruminants. Recently Corbett (1961) has again raised the question of how far palatability influences the selection of diet. The use of sheep with oesophageal fistulae has advanced the study of pasture intake by enabling one to find out what is eaten rather than what is left (Weir & Torell, 1959; Edlefsen, Cook & Blake, 1960).

Despite the wealth of physiological mechanisms that influence food intake, what is eaten is often limited in practice by what is available. Vercoe, Tribe & Pearce (1961) have shown that under Australian conditions the amounts of digestible organic matter and nitrogen eaten by sheep vary enormously with seasonal changes in the pasture, and this is reflected by seasonal changes in the microbial population of the rumen (Moir, 1951).

GAS IN THE RUMEN

Enormous volumes of gas are produced in the rumen. The rate of production has been measured both by drawing off gas from a rumen fistula and by use of a face mask and tracheal fistula (Colvin, Wheat, Rhode & Boda, 1957) and both methods gave rates of about 30 l/h from cattle on a diet of alfalfa. McArthur & Miltimore (1961) described a technique that allows a rapid and comprehensive analysis to be made by gas-solid chromatography on single small samples of gas. In agreement with earlier work they found rumen gas to contain about 65 % CO_2, 27 % CH_4, 7 % N_2, 0·6 % O_2, 0·2 % H_2 and 0·01 % H_2S.

The CO_2 is produced by bacterial metabolism and by release from salivary bicarbonate. The relative importance of these two sources depends partly on the diet and partly on the pH of the rumen, for as Cole, Huffman, Kleiber, Olson & Schalk (1945) have pointed out, saliva that is secreted at pH 8 in equilibrium with 6 % CO_2 will tend to absorb CO_2 when exposed to 70 % CO_2 in the rumen until its pH is reduced below about 6·9. The CH_4, H_2 and H_2S represent end products of reductive bacterial metabolism, and the N_2 and O_2 are either swallowed with the food or diffuse into the rumen from the blood. Almost complete anaerobiosis and a low redox potential are

maintained in the rumen as a result of rapid utilization of any oxygen that is added during feeding or rumination (Broberg, 1958), and the Eh is related linearly to the pH and so to the rate of bacterial metabolism (Baldwin & Emery, 1959).

Part of the CO_2 produced is absorbed through the rumen epithelium and exhaled by the lungs. The amount absorbed depends on the CO_2 tension in the rumen. In anaesthetized sheep CO_2 diffuses freely across the rumen epithelium and accumulates in saline solutions placed in the rumen (Ash & Dobson, 1963), but since the CO_2 tension in normal rumen contents is about ten times that in the blood the net movement of CO_2 will be from rumen to blood. Part of the methane produced is also absorbed from the rumen and exhaled (Cresswell, 1960; Waldo & Hoernicke, 1961).

Gas that is not absorbed is discharged from the rumen by belching. Not all of this is completely expelled for the glottis remains open during belching (Colvin *et al.* 1957; Dougherty, Hill, Campeti, McClure & Habel, 1962) and the high oesophageal pressure causes some of the gas to penetrate into the lungs where it is absorbed in amounts that may be of consequence to the animal (Dougherty, Stewart, Nold, Lindahl, Mullenax & Leek, 1962). Belching is readily stimulated by distension of the rumen with gas and cattle can dispose of 150 l of gas in half an hour when the rumen is inflated experimentally (Cole, Mead & Kleiber, 1942). The 'eructation contraction' of the rumen and its association with other contractions of the rumen has recently been described by Reid & Cornwall (1959) and by Stevens & Sellers (1959). Dougherty, Habel & Bond (1958) found that inflation of a pouch made from the reticulum and the cardiac region of the rumen was sufficient to stimulate belching in decerebrate sheep.

Bloat occurs when the belching mechanism fails to keep up with the production of gas, and the subject has been reviewed by Johns (1958) and Cole & Boda (1960). It is almost always due to bubbles of gas becoming trapped in a stable froth which inhibits the belching reflex. Consequently, although it can be prevented by depressing gas production with antibiotics, it is not necessarily associated with diets that give rise to the greatest volumes of gas. It is uncertain why froth formation sometimes occurs in the rumen, or why some animals are more prone to this condition than others. Plant saponins (Ferguson & Terry, 1955; Mangan, 1959), pectins and hemicelluloses (Head, 1959), proteins (Mangan, 1959; Bartley & Bassette 1961), and bacterial slime (Hungate, Fletcher, Dougherty & Barrentine, 1955; Gutierrez, Davis & Lindahl, 1958) form stable froths at various pH values, or are found in rumen froth, and all have been supposed to be at least partly responsible for various forms of bloat.

Saliva has been claimed to be an animal factor influencing bloat, but at present opinion is divided nicely as to whether it may prevent bloat or be its cause. Weiss (1953) first suggested that increased salivation would alleviate bloat by reducing the viscosity of the rumen contents. Johns (1958) countered with the suggestion that salivary bicarbonate might add to the gas released in the rumen and that salivary mucus might increase the frothiness of the contents. This was supported by work demonstrating the formation of stable froths by salivary mucins (Mangan, 1959) and the presence of certain muco-proteins (Lyttleton, 1960) in higher concentrations in the saliva of animals prone to bloat than in non-bloaters. In anaesthetized animals slight rises in rumen plus oesophageal pressure were shown to stimulate salivary secretions (Phillipson & Reid, 1958). This response was due to distension of the oesophagus and was inhibited by high pressures in the rumen corresponding to

moderate or severe bloat (Kay & Phillipson, 1959) so that stimulation of salivation could only occur in mild bloat, and then only if the cardiac sphincter relaxed sufficiently to allow froth to enter the oesophagus. More recently the role of saliva has been again reversed by Van Horn & Bartley (1961) and Bartley & Fina (1961) who showed that mucins from saliva and other sources release gas from frothing rumen contents and delay the onset of bloat, while mucinolytic organisms encourage bloat.

The surface tension of saliva is around 47 dynes/cm, compared with 71 dynes/cm for water. This perhaps is one of the main reasons for the low surface tension of rumen fluid (Reid & Huffman, 1949; Blake, Allen & Jacobson, 1957) and so may help to control the frothing of normal rumen contents. Plant lipids may also be important natural antifrothing agents (Fraser, 1961). The low surface tension of saliva and rumen fluid may have other important effects such as the promotion of rapid wetting and digestion of food (Bailey, 1962) and emulsification of fats (Hobson & Mann, 1961*b*), and it may also limit or encourage bacterial growth and activity, as was suggested by Reid & Huffman (1949). In addition salivary mucin has been shown to stimulate the growth of bacterial cultures *in vitro* (Gordon & Moore, 1961).

REGULATION OF THE VOLUME, COMPOSITION AND pH OF THE RUMEN FLUID

The consumption of water and salts and the secretion of saliva normally maintain the volume of rumen fluid and the flow of digesta onward from the rumen. In addition, together with absorptive mechanisms, they counter-balance the effects of microbial metabolism so as to keep the pH, solute concentrations and osmotic pressure of the rumen fluid within the fairly wide limits favourable to normal rumen function. The mechanisms regulating water and salt intake and salivary secretion will be considered first.

Water intake

The water economy of farm animals has been reviewed by Leitch & Thompson (1944). Water intake by cattle depends on a number of factors such as environmental temperature, the requirements of growth, pregnancy and lactation, and food intake. For every kilogram of dry matter eaten roughly 2–4 kg of water are drunk. When the amount of water supplied is restricted (Balch, Balch, Johnson & Turner, 1953; Phillips, 1961*b*; Wilson, 1963*b*, *c*) or water is provided only at 48 or 72 h intervals (French, 1956) the food intake falls. Balch *et al.* (1953) restricted the water intake of a fistulated cow to 70% of her normal intake. After a week of adjustment to the new régime, the weight of the rumen contents fell to about the same extent as the food intake and both the ratio of water to dry matter in the rumen and the digestibility of the food were more or less maintained. Phillips (1961*b*) compared the reaction of zebu and Hereford steers to a 50% reduction in water intake and showed that the slight fall in the intake of food was accompanied by increases in the time it was retained in the gut and in its digestibility. The percentage of dry matter in the rumen contents was little altered and no consistent changes in rumen fermentation rates were noted. The zebus were less affected than the Herefords and generally seemed to be better adapted to low water intakes, possibly as a result of greater salivary secretion. Despite the clear connection between food intake and water consumption the fluidity of the rumen contents probably depends more on the fairly

constant relationship of saliva secretion to dry-matter intake (see p. 275). Balch (1958) found that even during the course of a meal, when most drinking is done, the amount of saliva secreted was quite as large as the amount of water drunk. Over the 24 h, Bailey (1961*a*) found the estimated volume of saliva secreted was about three to five times greater than the volumes of dietary water plus drinking water consumed, so that the amount of water supplied to the rumen, and its ratio to dry matter intake, depended much more on the different salivary secretion rates for each diet than on the water intakes.

The dryness of the food in the mouth seems to be one of the main stimuli to drinking, for cattle drink frequently while eating hay so that water and hay consumption run in parallel (Balch, 1958). The cows studied by Bailey (1961*a*) tended to drink more on dry foods and silage than on fresh grass and more water was consumed by cattle eating cut grass when the grass matured and increased in dry matter content (Halley & Dougall, 1962). The osmotic pressure of the blood is also important. Beilharz & Kay (1963) found that sheep became very thirsty when they were given intravenous injections of hypertonic NaCl solutions, but not when given hypertonic glucose solution. Apparently, as in other species, the sheep's osmoreceptors respond selectively to solutes which do not penetrate the cell membrane readily. On the other hand, the osmotic pressure of the rumen contents appears to play no part, for Beilharz & Kay's sheep showed no sign of thirst when hypertonic $NaHCO_3$ solutions were introduced into the rumen. Sheep depleted of sodium by loss of saliva continue to drink water although the concentration of sodium in their plasma is falling (Beilharz, Denton & Sabine, 1962), so that a probably low plasma osmotic pressure does not fully inhibit thirst any more than it does in men depleted of sodium by sweating.

Thirsty sheep (Beilharz *et al.* 1962), like thirsty dogs (Bellows, 1939), drink in a few minutes the amount of water needed to rehydrate themselves and then appear satisfied although little water can have been absorbed from the gut in this time. In dogs temporary satiation appears to be given by pharyngo-oesophageal receptors that meter the water intake. Andersson & McCann (1955) have located a 'drinking centre' in the lateral hypothalamus of goats whose stimulation is accompanied by rapid and continuous drinking. Similar centres are found in other mammals (Anand, 1961).

Salt intake

Most plant foods contain large amounts of potassium but rather little sodium. Green roughages and grains generally contain less than 0·1 % of sodium (40 m-equiv./kg wet weight) and some less than a tenth of this amount, and dry roughages contain from 0·1 to 0·5 % sodium (40–200 m-equiv./kg) (Morrison, 1956). In arid countries there may be still less sodium in the forage; for example, Denton, Goding, Sabine & Wright (1961) quote sodium contents of 1–20 m-equiv./kg for various dry fodders fed to stock in Central Australia. The ratio of sodium to potassium in the plant reflects that in the soil and Stewart & Holmes (1953) have shown that the sodium yielded by grassland is progressively reduced by repeated application of KCl.

The well-known liking for salt shown by cattle and sheep may be related to the scarcity of sodium in the food. Denton and his colleagues in Melbourne have studied

the appetite for sodium in sheep under a variety of conditions. Denton (1957*a*) found that sheep that are depleted of sodium by loss of saliva from a parotid fistula licked large amounts of sodium off salt blocks. Normal sheep with adequate sodium intakes showed a liking for sodium solutions, especially NaCl, consuming up to 700 m-equiv. of sodium daily (Denton & Sabine, 1961). The sheep were then depleted of sodium by construction of a parotid fistula and they increased their intake of the sodium solutions, if low previously, until they achieved sodium balance again. Parotid saliva has a high bicarbonate content and so it was interesting to find that after fistulation the sheep's preference switched from NaCl to $NaHCO_3$. The sheep were able to balance their sodium deficit even when the solution was offered for only an hour or less daily, or when the concentration was varied over an eightfold range. Most of the sodium consumed was drunk within 5–10 min of being offered although little can have been absorbed in this time. Sodium-depleted calves also show an increased appetite for sodium solutions (Bell & Williams, 1960).

The stimulus for this specific appetite for sodium was sought in the changes that accompany sodium depletion. The concentration of sodium in the rumen falls during sodium depletion but this does not serve as the stimulus; Beilharz & Kay (1963) found that depleted sheep drank as much sodium 10 min after pouring 1·5 l of hypertonic $NaHCO_3$ solution into the rumen as they did after pouring tap water into the rumen. The absolute concentration of sodium in the plasma is not the stimulus either. Sheep that are depleted of both sodium and water, so that the concentration of sodium in the plasma is unaltered or even raised, still show appropriate and specific appetites for both water and sodium solutions (Beilharz *et al.* 1962). Moreover, intravenous infusions of hypertonic glucose which expanded the blood volume and reduced the concentration of sodium in the plasma by about 10 m-equiv./l did not affect the sodium appetite of sodium-depleted sheep when this was tested 10 min later (Beilharz & Kay, 1963). When hypertonic NaCl solutions were infused the concentration of sodium in the plasma was raised by some 20 m-equiv./l, again with an expansion of blood volume, and although the sodium appetite was usually reduced this did not always happen and the appetite bore no relation to the current concentration of sodium in the plasma.

The sheep thus seems able to assess roughly the total amount of sodium in its extracellular fluid and trims its sodium appetite appropriately, but it is not clear how this is done. The lack of effect of glucose infusion would be expected if the appetite for sodium is determined by a mechanism which measures both plasma sodium concentration and blood volume, but this cannot account for the continued existence of the appetite following the NaCl infusion. Beilharz *et al.* (1962) suggest that the sheep responds to a concentration gradient for sodium between an intracellular and an extracellular compartment; water depletion might affect both compartments equally and so not alter the gradient.

Salivary secretion

Ruminants secrete enormous volumes of alkaline and well-buffered saliva. This not only moistens the food in the mouth, aiding chewing and swallowing as in other mammals, but also stabilizes the rumen pH and supplies most of the water and salts of the rumen fluid. Sheep are estimated to secrete 6–16 l daily (Kay, 1960*a*) and

cattle as much as 98–190 l (Bailey, 1961*a*). These are considerably greater than earlier estimates which have been summarized by Somers (1957) and Bailey (1961*a*).

Kay (1960*a*) divided the salivary glands of sheep into three groups. First, the parotid and inferior molar glands which are purely serous, secrete continuously and may be stimulated reflexly from the mouth, oesophagus and rumen. They are particularly active during eating and rumination. The saliva is not at all mucous, and is isotonic with plasma, alkaline and well buffered. The small glands lying beneath the epithelium of the cheeks, palate and pharynx form the second group. They are composed of large mucous cells and together weigh about 20 g. They secrete little when not stimulated but like the serous glands they respond well to stimulation of the mouth, oesophagus and rumen. Palatine saliva is extremely mucous but resembles parotid saliva in its inorganic constituents and alkalinity. The third group consists of the submaxillary, sublingual and labial glands; these contain both small mucous cells and some serous cells. The submaxillary gland secretes rapidly during feeding but only slightly during rumination and it is little affected by stimulation of the oesophagus and rumen (Kay, 1958; Kay & Phillipson, 1959; Ash & Kay, 1959). Submaxillary, sublingual and labial salivas are mucous, hypotonic and poorly buffered (Kay, 1960*a*).

The parotid gland has been studied most extensively. Eckhard (1893) showed long ago that the gland continues to secrete when denervated, and this flow persists even when massive injections of atropine are given or when the gland is isolated and perfused (Coats, Denton, Goding & Wright, 1956; Kay, 1958). This suggests that the continuous secretion is due to some property of the gland itself, as claimed by Eckhard (1893). Krinitsin (1940), on the other hand, was impressed by the slowness of parotid secretion in unweaned calves and in fasted adult ruminants and held that the secretion of the denervated gland is stimulated by continuously absorbed products of digestion. This possibility is not wholly ruled out by perfusion experiments of short duration. This basal component of parotid secretion ensures that the flow of saliva to the rumen never ceases altogether but it probably accounts for only about one-eighth of the total volume of parotid saliva secreted (Kay, 1958).

Recent measurements of the volume of saliva secreted by single parotid glands range from 1 to 5 l daily in stall-fed sheep (Marston, 1948; Denton, 1957*a*; Somers, 1957; Stewart & Dougherty, 1958; Kay, 1960*a*; Wilson, 1963*a*, *b*, *c*) and may reach 6–8 l daily in sheep at pasture (Wilson, 1962, pers. comm.). In cattle, volumes of 22–80 l daily have been recorded (Blokh, 1939; Bailey & Balch, 1961*a*; Dobson, 1963). The volume of saliva secreted by single parotid glands varies surprisingly in otherwise similar sheep. Kay (1960*a*) showed that only a small part of the variation was due to differences in body weight. The two parotid glands of a sheep sometimes differed in weight by up to 50%; presumably their secretion rates differed similarly and this points to the danger of doubling the secretion of one gland in order to obtain an estimate of total parotid secretion.

Artefacts arising from the collection technique may be another source of variation. Denton (1957*a*) has described how the parotid papilla may be transplanted to a skin teat hanging from the cheek. When the operation is successful the gland remains normal in chemical composition and histological appearance and it weighs about as much as the unfistulated gland. On the other hand, if the duct becomes obstructed

temporarily part of the gland is destroyed and this may account for some of the lower secretion rates found in such preparations. Wilson (1963*a*) found that 3 weeks after the parotid nerve and duct were cut on one side, causing complete fibrosis of the gland, the opposite parotid and other salivary glands secreted more rapidly. One cannot be sure whether this effect was due to destruction of the gland itself or to the absence of its secretion. If the latter then the loss of saliva that follows creation of a parotid fistula may cause some degree of compensatory oversecretion and perhaps growth by both the fistulated and normal glands. Saliva may also be collected by cannulating the cut parotid duct but this may easily damage the fine parotid nerve fibrils that run beside the duct. Such an accident may account for the poor responses to feeding, etc., obtained by some earlier workers.

The volume of saliva secreted depends to a large extent on the quantity and nature of the food eaten. Denton (1957*a*) recorded that a sheep secreted 3·17 l of parotid saliva daily when eating 0·8 kg of lucerne chaff and 0·3 kg of fresh lucerne but only 0·5 l when fed fresh lucerne alone, although the intake of 2–3 kg daily supplied about as much dry matter as the first diet. Starvation reduced the parotid secretion to 0·3–0·5 l after 4–5 days and restriction of the water intake or severe sodium depletion also reduced secretion to 0·5 l or less. On the other hand, Wilson (1963*b*) found that as much parotid saliva was secreted by sheep on fresh grass as on dried grass containing the same amount of dry matter. The amount of parotid saliva secreted when chaffed hay was fed at three levels of intake was linearly related to the dry-matter intake, roughly 3 l being secreted by a single gland for every kilogram consumed. The volume secreted was not affected by increasing or restricting the water intake but was greatly reduced when 10 l of artificial saliva or 1 % NaCl solution was infused into the rumen daily. The physical nature of the diet was important in some respects. More saliva was secreted when the hay fed was ground to pass a $\frac{1}{16}$ inch screen than when it was fed in the long state, but very much less was secreted when still more finely sieved hay was given.

Wilson's results agree with earlier experiments on the secretion of mixed saliva by cattle. Balch (1958) collected and weighed swallowed food as it passed through the cardiac orifice in order to measure the weight of saliva added to it. Hay had three or four times more saliva added to it than concentrates or flaked maize, for although the rate of secretion was slightly higher with concentrates the hay was chewed for much longer before being swallowed. Fodder beet and fresh-cut grass had the least amounts of saliva added to them although, on a dry weight basis, the grass had as much saliva added as the hay. This work was extended by Bailey (1961*a*) who found that the rate of secretion of saliva by any one cow was much the same whether it was eating dairy cubes, hay, dried grass or silage, and was only lower on mangolds and one cut of fresh grass. Consequently the amount of saliva added to the food depended mainly on how long it was chewed. The total water content of all the swallowed foods except the dairy cubes was between 78 and 93 %; for dairy cubes it was only 50 %. Estimates of total daily salivary secretion, based on the rates of secretion during feeding, rumination and 'resting' and the duration of each activity, were greatest for diets of fresh grass, less for hay and least for high concentrates diets and silage. The relatively small amounts of saliva added to diets of meals and concentrates perhaps explains the thickness of the rumen contents found with such diets. Speaking

generally, the diet seems to influence salivary secretion principally by determining how long is spent in feeding and ruminating. The numerous reflex paths by which the physical consistency of the rumen contents might be expected to influence salivary secretion (see below) appear to be of subsidiary importance.

In Balch's and Bailey's experiments care was taken to avoid stimulating the sensitive cardiac region with the rubber cup used to collect swallowed material. Measurements of mixed salivary secretion have also been made by obstructing the oesophagus with a balloon (Hydén, 1958) or with an inflatable catheter drawn up from the cardia (Cunningham, 1958), and aspirating the saliva collecting above the obstruction through a nasal tube. Other workers have used a tube inserted into the oesophagus through the cardia (McGilliard, Conner, Duncan & Huffman, 1957; Emery, Smith, Grimes, Huffman & Duncan, 1960; Sasaki & Umezu, 1962). Unfortunately, as described next, distension of the cardia and lower oesophagus is a particularly strong stimulus to salivary secretion, as these authors recognized, and this renders the experiments of uncertain value so far as they concern measurements of salivary flow.

A large number of reflexes have been described which account for the increased flow of parotid saliva during eating and rumination. The parotids are stimulated by brushing between the tongue and molar teeth, a partly one-sided effect (Kay, 1958), and by injecting fatty acids into the mouth (Krasuskii & Krymskaia, 1940; Coats *et al.* 1956; Denton, 1957*a*); by brushing the lower oesophagus (Clark & Weiss, 1952) and by distending it with gas, fluid or balloons (Kay & Phillipson, 1959; Sellers & Titchen, 1959); by stretching the cardiac and reticulo-omasal orifices and the reticulo-rumen fold and by tactile stimulation of these and other structures of the reticulum and the anterior part of the rumen (Comline & Titchen, 1957; Kay, 1958; Ash & Kay, 1959). The sensory and motor nerves concerned in these reflexes are described by Kay (1958). The introduction of fatty acids into the rumen also stimulates parotid secretion, but the response is transient and the pH required is much lower than is normally found in the rumen so this effect is unlikely to play any part in the normal regulation of salivary secretion (Kay, 1958; Ash & Kay, 1959). Bailey & Balch (1961*b*) added 300 ml of acetic acid to the rumen contents of cows, lowering the rumen pH to 5·4, without altering the rate of salivary secretion.

Parotid secretion is also influenced by inhibitory stimuli. Elovskikh (1937*b*) first showed that parotid secretion is depressed in calves when the rumen is inflated or distended with water. In similar experiments on anaesthetized sheep and calves Phillipson & Reid (1958) and Kay & Phillipson (1959) inhibited salivary secretion by inflating the rumen to pressures of 20–30 mmHg or, under some conditions, to less than 9 mmHg. Parotid secretion was inhibited in conscious sheep when balloons were inflated to 1–2 l in the rumen (Ash & Kay, 1959). Bailey & Balch (1961*a*) found that the rate of parotid secretion fell during a meal and only recovered slowly during the following 8 h; total salivary secretion behaved similarly and was most reduced following the largest meals (Bailey & Balch, 1961*b*). They suggest that salivary secretion is inhibited by the act of feeding itself. Wilson (1963*c*) obtained similar results in sheep, and also found that parotid secretion was inhibited after a drink or after water or saliva was added to the rumen through a fistula. The removal of 1–2 l of rumen contents after a meal allowed a substantial increase in parotid secretion.

This, Wilson suggests, is because distension of the rumen with food, water and saliva during the feeding period provides an inhibitory stimulus which gradually declines as the volume of rumen contents diminishes between feeds.

Conditioned stimuli also affect salivary secretion. Denton (1957*b*) summarized previous Russian work on the subject; his own work showed that the usual activities that precede feeding cause sheep to secrete parotid saliva profusely. The preparation of a sodium solution in sight of a sodium-depleted sheep has a similar effect (Beilharz & Kay, 1963).

The rates of secretion of the remaining salivary glands have received much less attention. Kay (1960*a*) recorded flows of from 185 to 375 ml daily from single submaxillary glands of sheep on a diet of hay and meals. The sublingual glands of sheep are too small to secrete much saliva and it is not known how much the larger labial glands secrete. The substantial secretion of the remaining small glands of the mouth of sheep was first shown by Scheunert & Krzywanek (1929) who collected the mixed salivary secretions from a glass cannula tied into the oesophagus. After both parotids had been cannulated the residual flow of saliva from the oesophagus was as great as the flow of parotid saliva. Phillipson & Reid (1958) and Kay & Phillipson (1959) cannulated the parotid and submaxillary glands in anaesthetized sheep in which salivary secretion was stimulated, and they also found that as much saliva dribbled from the mouth and nose as was secreted by the parotids. This residual saliva consisted of mucous strands over which ran a more fluid component and clearly represented a mixture of the secretions of the small mucous glands and the serous inferior molar glands. Kay (1960*a*) estimated that the inferior molar glands of sheep secrete 0·7–2·0 l and the small mucous glands a further 2–6 l daily.

The alkaline nature of ruminant saliva has been recognized since Lassaigne's analysis of the parotid saliva of a cow and a ram (Lassaigne, 1852). McDougall (1948) made the first detailed analysis of sheep parotid saliva which contained, on average, in m-equiv./l: Na^+, 177; K^+, 8; HCO_3^-, 104; HPO_4^{2-}, 52; Cl^-, 17; and less than 1 m-equiv./l of Ca^{2+} and Mg^{2+}. The pH at room temperature was 8·23, corresponding to 8·09 in the saliva as secreted. Mixed saliva is very similar in composition, as also are 'residual' and palatine salivas (Phillipson & Mangan, 1959; Kay, 1960*a*), the mixed and parotid salivas of cattle (Emery *et al.* 1960; Bailey & Balch, 1961*a*, *b*) and the parotid saliva of goats (Kay, 1960*b*).

The two most important factors that affect the composition of parotid saliva are sodium depletion and the rate of secretion. Denton (1956) first described the remarkable inversion of the relative concentrations of sodium and potassium in parotid saliva that occurs when sheep are depleted of sodium. In one sheep, for instance, the concentration of sodium fell from 178 to 46 m-equiv./l and that of potassium rose from 21 to 140 m-equiv./l after nearly 1000 m-equiv. of sodium had been lost from a parotid fistula, at which stage a new balance between the dietary sodium intake and the reduced salivary loss was achieved (Denton, 1957*a*). This effect is due to aldosterone, secreted by the adrenal glands under the stress of sodium depletion, and has been fully reviewed by Denton, Goding & Wright (1960). Sodium depletion also reduces the sodium : potassium ratio of submaxillary saliva, but has little or no effect on the small mucous glands which probably continue to secrete considerable amounts of sodium into the rumen even in acute sodium depletion (Kay, 1960*a*). An increase

in the rate of secretion of parotid saliva typically increases the concentrations of sodium and bicarbonate and decreases the concentrations of potassium and phosphate to a reciprocal extent (Coats & Wright, 1957; Kay, 1960*a*; Bailey & Balch, 1961*a*). This effect is most noticeable in the high-potassium saliva secreted during sodium depletion. The concentration of phosphate in parotid saliva is fairly closely related to its concentration in jugular blood (Watson, 1933) and may be increased by supplementing the diet with phosphate (Denton, 1957*a*).

Submaxillary saliva is only weakly buffered (Lassaigne, 1852). Kay (1960*a*) gave the average concentration of electrolytes in the submaxillary saliva collected over 24 h periods, in m-equiv./l, as Na^+, 9; K^+, 16; HCO_3^-, 9; HPO_4^{2-}, 5; Cl^-, 11. Sublingual and labial salivas were similar. The concentrations of calcium and magnesium in 'residual' and submaxillary salivas are approximately twice what they are in parotid saliva (Storry, 1961). The composition of submaxillary saliva varies with the rate of secretion and this probably accounts for differences between samples collected from anaesthetized and conscious sheep (Phillipson & Mangan, 1959; Kay, 1960*a*).

The organic constituents of saliva, principally mucus and urea, may be of importance in bloat (p. 270) and are a source of nitrogen to the micro-organisms of the rumen (p. 287). Urea accounts for most of the nitrogen present in parotid saliva and its concentration is proportional to but rather lower than its concentration in plasma (see p. 287). Submaxillary saliva contains much more protein nitrogen than parotid saliva (Phillipson & Mangan, 1959). The hexosamine and mucoprotein contents of mixed saliva have been studied by Emery *et al.* (1960) and Lyttleton (1960). Volatile fatty acids are present in saliva at about their concentration in the blood (Annison, 1954).

Although ruminant saliva contains little or no diastase (see Mangold, 1929, p. 120), milk that is sham-fed to calves undergoes some lipolysis (Wise, Miller & Anderson, 1940). This is probably due to lipolytic enzymes secreted by the submaxillary gland and certain buccal and oesophageal glands (Ramsey, Wise & Tove, 1956; Ramsey & Young, 1961). Less lipolytic activity was found in adult cattle than in calves (Ramsey *et al.* 1956) and none was found in saliva taken from adult sheep (Garton, Hobson & Lough, 1958).

Production of volatile fatty acids in the rumen

Most of the digestible carbohydrate fed to ruminants, including cellulose, is fermented to steam-volatile fatty acids in the rumen. Additional fatty acids, including branched-chain forms, are produced by deamination of amino acids. The production and absorption of fatty acids have recently been reviewed by Annison & Lewis (1959), Barnett & Reid (1961), Blaxter (1961, 1962) and Dobson (1961) and the position need only be briefly summarized here.

Blaxter (1961) has tabulated estimates of the amounts of fatty acids absorbed daily by sheep on various diets. From 0·77 to 2·52 moles of acetic acid are absorbed, together with 0·27–0·72 mole of propionic acid and 0·11–0·69 mole of butyric acid. The total amount of fatty acid absorbed by sheep on maintenance rations is probably a little over 3 moles daily (Blaxter 1962, p. 212). Cattle produce about ten times this amount. D. A. Balch (1958) estimated the production of volatile fatty acids in lactating cows by multiplying the weight of food digested in the rumen, measured in

samples taken from near the reticulo-omasal orifice, by the amounts of fatty acids that would be produced by this degree of digestion, measured by an *in vitro* technique. This gave production rates of 1810–3870 g (about 25–55 moles) daily for diets of hay and concentrates. Stewart *et al.* (1958) multiplied fermentation rate, measured *in vitro*, by rumen fluid volume, measured directly, and arrived at similar figures for steers, corresponding to about 42–57 moles daily.

The concentration of total volatile fatty acids in the rumen depends on the feed intake. Williams & Christian (1956) found the concentration to fall from 97 to 71 mM/l as the dried grass intake of sheep was reduced from 1000 to 400 g daily. The concentration also depends on the nature of the diet; it is roughly 150 mM/l in animals grazing freely or fed rations high in concentrates, about 100–150 mM/l in those fed hay or silage, and is usually less than 100 mM/l when large amounts of maize are fed. Annison & Lewis (1959, p. 61) listed some of the results given by various authors. The molecular proportions of the acids also vary. Acetic acid usually accounts for the majority of the fatty acid present, with propionic, butyric and valeric acids contributing progressively smaller fractions, but the relative importance of acetic acid is rather less on high food intakes (Williams & Christian, 1956). Diets rich in starch reduce the pH of the rumen contents and increase the proportion of propionic to acetic acid. Balch & Rowland (1957) and Reid, Hogan & Briggs (1957) argued that the acid conditions may favour a microbial population producing an unusually high proportion of propionic acid. This suggestion is not supported by the work of Emery & Brown (1961) who found that grain diets were associated with a low molar proportion of acetic acid in the rumen whether the rumen pH was low or was raised to 6·3 by addition of bicarbonate supplements.

Lactic acid is an intermediary in the breakdown of a small fraction of carbohydrate to fatty acids (Jayasuriya & Hungate, 1959) and little is found in the rumen on most diets (see p. 305). When large amounts of starch or sugar are fed the rate of production of lactic acid exceeds its rate of dissimilation so that the concentration builds up to about 70 mM/l (Phillipson, 1952) or higher (Briggs, Hogan & Reid, 1957) and the pH of the rumen contents falls below 5. Other intermediary fatty acids, including formic acid, may sometimes be found in vanishingly small concentrations and they disappear rapidly when added to the rumen.

Fermentation of the food material leaving the rumen is arrested by acidification as it passes through the abomasum. Fatty acids are again formed by microbial action in the alkaline environment of the lower ileum and large intestine. In ruminants this second attack on the food residues is probably of little quantitative importance but in non-ruminant herbivores the large intestine serves as the main vessel for microbial digestion (Hungate *et al.* 1959).

Regulation of pH in the rumen

The pH of the rumen contents is usually between 5·5 and 7·3. Lower pH values are almost always associated with the production of lactic acid in the rumen. Within the normal range the pH depends principally on the balance achieved between production and absorption of volatile fatty acids and the buffering power of the rumen contents. Fatty acid concentration and buffering power vary independently so that a clear correlation between pH and the concentration of volatile fatty acids is seldom seen

except in the reciprocal fluctuations of the values that occur after a meal (Phillipson, 1942). In fact Williams & Christian (1956) found that when the concentration of volatile fatty acids in the rumen was lowered by reducing the amount of food eaten there was no change in the rumen pH at all. The relationship of pH to volatile fatty acid concentration varies between animals and between diets (Balch & Rowland, 1957; Briggs, Hogan & Reid, 1957) and considerable daily and seasonal variations in pH may occur, at least under Western Australian conditions, even though the food intake is kept nearly constant (Nottle, 1956).

The acidity of the rumen contents influences the rate and course of microbial metabolism; recent work on this topic is reviewed in Part 2. A low rumen pH delays the establishment of the protozoal population in calves (p. 290) and greatly reduces the ciliate count in sheep (Purser & Moir, 1959). When diets containing large amounts of sugar or starch are fed a lactic fermentation develops in the rumen, the pH drops and rumination and reticuloruminal contractions are depressed and may stop altogether (Hungate, Dougherty, Bryant & Cello, 1952; Scarisbrick, 1954). Acetic acid has similar effects when injected into the rumen (Matscher, 1958). Ash (1959) poured acid solutions into the empty rumen of conscious sheep and found that reticulum contractions were reflexly inhibited by acetic, propionic and butyric acids at pH 3·6–5·0, by lactic acid at pH 2·5, but not by citric or hydrochloric acids at still lower pH values. He suggested that receptors are present in the wall of the rumen which are stimulated not so much by pH of the rumen contents as by the concentration of unionized volatile fatty acids; these can reach the postulated receptors by rapid penetration of the rumen epithelium.

When urea is given into the rumen as a drench it is swiftly hydrolysed by bacterial action; the pH and concentration of ammonia in the rumen rise and are associated with symptoms of urea poisoning. Coombe *et al.* (1960) showed that rumination declined when the pH rose above 7·0 and rumen movements stopped altogether at 7·3. Le Bars & Simonnet (1959) were able to match the symptoms of urea poisoning by giving intravenous injections of ammonium acetate but not by injecting urea. They therefore suggested that urea poisoning is caused by ammonia after its absorption from the rumen. A high rumen pH in itself has almost no effect on rumen motility (Ash, 1959), neither has a high rumen ammonia concentration by itself; rumen ammonia may safely reach high concentrations when protein-rich diets are fed since the simultaneous release of fatty acids neutralizes the ammonia and prevents more than a fractional increase in pH (Briggs, Hogan & Reid, 1957). Hogan (1961) has shown that ammonia is absorbed from the rumen either as unionized NH_3, or as NH_4^+ in association with acetate. The pK value for ammonia in solution is high, about 9·3, so that the proportion of total ammonia present as NH_3 will increase as the rumen pH rises. Coombe *et al.* (1960) suggested that only when both the total ammonia concentration and the pH in the rumen are high will the concentration gradient forcing NH_3 from rumen to blood cause such rapid absorption of ammonia that some evades conversion to urea by the liver and passes into the peripheral circulation. The relation of rumen ammonia concentrations to blood ammonia and urea levels and the acid-base status of the blood were discussed further by Annison & Lewis (1959) and by Lewis (1961).

Turner & Hodgetts (1955) showed that sheep rumen contents are well buffered

against addition of acid but poorly buffered against alkali. Bicarbonate and phosphate account for most of the buffering power within the normal pH range of the rumen and were almost equally important. Below pH 5·5 the buffering is largely due to fatty acids. Particulate matter has little buffering action. The importance of saliva as a source of the buffers present in the rumen has often been stressed for it supplies a large amount of bicarbonate and almost all of the inorganic phosphate found in the rumen. The total amount of phosphate secreted daily in the saliva of cattle, for example, based on measurements of the volume and composition of mixed saliva given by Bailey (1961*a*) and Bailey & Balch (1961*b*), is from 2·5 to 5·0 equiv., and the total amount of bicarbonate secreted is from 12 to 24 equiv. (1–2 kg of sodium bicarbonate) a great increase on Markoff's earlier estimate of 300 g of sodium carbonate (Markoff, 1913). Impressive as these quantities are, they are not sufficient to neutralize all the fatty acid produced; Ash & Dobson (1963) have calculated that the amount of alkali secreted in the saliva of sheep is only enough to raise about one-third of the fatty acids formed in the rumen to the normal pH of about 6·5. The buffers secreted in the saliva, together with any buffers derived from the food, may therefore represent only a first line of defence against excessive acidity in the rumen, neutralizing the spurts of acid produced after feeding, while the bulk of the acid is removed by absorption (see below).

The part played by saliva is perhaps over-emphasized by McManus (1962) who found that when saliva is prevented from entering the rumen after a meal the volatile fatty acid concentration in the rumen rises and the pH falls to greater extents than are found in the conscious and unrestrained sheep. Since all but one of the sheep used had to be either anaesthetized or gagged and held on one side in order to remove the saliva, it is not possible to say whether the abnormal conditions in the rumen were due wholly to loss of saliva or whether some unmeasured alteration, such as a reduction in rumen blood flow and fatty acid absorption, or in the mixing and outflow of the rumen contents, also influenced the results. This criticism does not apply to the one experiment in which saliva was removed from a sheep through an oesophageal fistula, and further experiments of this kind should give interesting results. In the experiments of Bailey & Balch (1961*b*) the pH of the rumen contents on various diets did not seem to be related to the rates of secretion of saliva at all, but this may have been because other variables, such as fatty acid production, obscured the relationship.

Fatty acids that are not neutralized by saliva are mostly neutralized by the blood after being absorbed through the rumen wall in their associated acid form. Fatty acids pass through the rumen epithelium in either direction down their concentration gradients. Their absorption has been reviewed by Dobson (1961). Danielli, Hitchcock, Marshall & Phillipson (1945) first showed that when volatile fatty acids are introduced into the isolated rumen at a pH below 6 they are absorbed predominantly in the associated state and more acid has to be added to prevent the pH from rising. This indicates a way in which acid absorption, together with salivary buffers, could hold the pH of the rumen between 5 and 6, as occurs when carbohydrate-rich diets are fed. At first sight, however, it does not readily explain how on roughage diets the pH can be held above 6, at which level the fatty acids are largely ionized. Fatty acids are absorbed more slowly at a high pH, and Danielli *et al.* (1945) suggested that

this is because the rumen epithelium is less permeable to ionized fatty acid radical than to associated fatty acid. Masson & Phillipson (1951) found that fatty acids were absorbed from alkaline solutions placed in the isolated rumen, and at the same time bicarbonate and chloride appeared in the solution, one molecule of bicarbonate replacing two of fatty acid. This suggests that bicarbonate may be secreted into the rumen and Ash & Dobson (1963) have shown that the rumen epithelium is indeed permeable to both CO_2 and bicarbonate, which diffuse down their chemical or electrochemical gradients in either direction, but is only negligibly permeable to hydrogen ion. They suggest, nevertheless, that most of the bicarbonate that appears in the rumen when fatty acids are absorbed is not secreted into the rumen as such, but that dissolved CO_2 diffusing into the rumen dissociates into bicarbonate and hydrogen ion; the hydrogen ion then combines with fatty acid radicals and is absorbed as free fatty acid. In the intact sheep, of course, the CO_2 would be produced within the rumen. If this is the case, it is a way in which the rumen epithelium may remove large amounts of fatty acids in unionized form by absorption even at fairly high pH values. Fatty acid that is absorbed in the ionized state is largely balanced by Na^+ absorption.

As the rumen pH falls the amount of acid that can be neutralized by saliva and the rate of acid absorption are likely to increase. Increased absorption of acid will also stimulate the blood flow to the rumen (Dobson & Phillipson, 1956). The disorders of the rumen that occasionally develop when highly digestible and finely divided diets are eaten nevertheless indicate a failure to deal adequately with the increased formation of acid in the rumen, and the addition of sodium bicarbonate or other alkali to the diet is sometimes beneficial. Some of the extensive work on this subject is referred to in recent papers by Nicholson, Cunningham & Friend (1962). More precise measurement of the buffering power of the rumen fluid may help to define some of the problems associated with intensive feeding.

Lactic acid is not likely to be absorbed from the rumen at all rapidly. Its lipid solubility is lower than that of acetic acid and its pK is almost one unit lower (about 3·8) so that very little unionized lactic acid will be present even at the low rumen pH values found during acid indigestion.

Hueter, Shaw & Doetsch (1956) found that blood lactate concentrations began to rise sharply so soon (half an hour) after placing large amounts of sodium lactate, etc., into the rumen that they suggested that some of the lactate was absorbed directly from the rumen. A more likely explanation seems to be that the lactate was absorbed lower down the digestive tract, for Dobson & Phillipson (1956) could find no evidence for absorption of lactate from the isolated rumen using solutions buffered at pH 4. Absorption of lactate ion will not help to reduce the acidity of the rumen contents, of course.

Smith, Kleiber, Black & Baxter (1955) measured the secretion of isotopic phosphorus into the rumen contents and argued that the amount transferred was greater than could be fully accounted for by salivary secretion (which was not independently measured). They therefore proposed that substantial amounts of phosphate must enter the rumen through the rumen epithelium. This seems most unlikely, for both concentration and potential gradients strongly oppose such a movement of phosphate through the rumen epithelium which in any case is only very slightly permeable to this ion in either direction (Hydén, 1961*c*; Ash & Dobson, 1963). Happily, the upward

revision of salivary secretion rates since 1955 makes it possible to account for the phosphate turnover rates of Smith *et al.* (1955) without invoking secretion of phosphate by the rumen.

Osmotic pressure and salt concentrations in the rumen

The osmotic pressure of the rumen contents and the passage of water through the rumen epithelium have received little attention, although they may well influence the volume of the rumen contents and the outflow of rumen fluid to the omasum (Murray *et al.* 1962). The work of Parthasarathy & Phillipson (1953) suggests that the rumen contents tend towards isotonicity with the blood. Solutions that were hypotonic or isotonic to serum lost water when put into the isolated rumens of anaesthetized sheep while hypertonic solutions gained water. Solutes were also absorbed from all the solutions in amounts that increased with the tonicity, but this did not obscure the fact that water moved across the rumen epithelium as a result of osmotic gradients. Further observations showed that the rumen contents of sheep on diets of hay or hay and oats had osmotic pressures within about 20 % of that of serum, although a maintained rise within this range occurred after feeding linseed meal. Three mechanisms may thus account for the near isotonicity of rumen contents: the permeability of the rumen epithelium to water, the absorption of salts by the rumen epithelium, and the secretion of large volumes of nearly isotonic saliva.

If the rumen contents are to remain isotonic any solutes absorbed, other than fermentation products, must be accompanied by an appropriate volume of water. It is interesting to note that Hydén's estimate of the rate of absorption of water from the rumen contents, 0·15 l/h (Hydén, 1961*c*), corresponds to 3·6 l daily and is enough to render roughly isotonic the 600 m-equiv. of sodium (plus anion) that Dobson (1959) estimated is absorbed from the rumen. Volatile fatty acids and other fermentation products that are continuously formed in the rumen and absorbed from it would be expected to have little long-term effect on the osmotic pressure and water balance of the rumen; intermittent surges of fermentation may have more substantial effects. Unabsorbed fatty acids will only increase the number of osmotically active particles in solution to the extent that they are not neutralized or are neutralized by HPO_4^{2-} to give $H_2PO_4^-$ plus acid radical, for neutralization by HCO_3^- is accompanied by the evolution of gaseous CO_2.

If the isotonicity of the rumen contents is due mainly to the permeability of the rumen epithelium to water, then the rate at which rumen fluid passes on to the omasum will depend more on the amount of salts that enter the rumen than on the water intake. Hints that this may be the case are provided by observations that the rate of passage of food through the rumen is accelerated by including large amounts of NaCl in the diet (Elam, 1961), and, conversely, that the addition of 4 or 8 l of water to the rumen daily causes only very slight increases in food intake (Lloyd Davies, 1962). On most dry diets saliva will supply most of the salts entering the rumen so that the onward flow of rumen fluid will reflect the rate of salivary secretion. Absorption of sodium by the rumen epithelium will lessen this effect of saliva, especially when green forages containing large amounts of potassium are eaten (see p. 284).

The concentrations of individual electrolytes in the rumen contents depend largely on the amounts of salts and water that are ingested, the amounts secreted in the

saliva, and the amounts absorbed by the rumen epithelium. Dobson & Phillipson (1958) and Dobson (1959) have measured the movements of sodium, potassium, chloride and water across the epithelium of the isolated rumen in anaesthetized sheep. Electrolyte movements were affected not only by the permeability of the epithelium and the concentration gradient, but also by the potential difference between rumen contents and blood, in which blood is normally about 30 mV positive. Sodium is absorbed in large amounts despite an unfavourable electrochemical gradient, so that some other force must be acting upon it. Since the absorption of sodium is balanced electrically by absorption of acetate to a considerable extent, the factors that govern the amount of acetate absorbed (concentration, pH, etc.) may also influence sodium absorption; this possibility calls for exploration. Potassium is absorbed in much smaller amounts down a favourable electrochemical gradient. Chloride passes through the rumen epithelium in either direction down its electrochemical gradient, but may enter the rumen slightly against its electrochemical gradient when the potassium concentration is high. The electrochemical gradient only favours chloride absorption when the concentration of chloride in the rumen exceeds about 30 m-equiv./l, which seldom happens, but in fact Hydén (1961*c*) observed net chloride absorption at lower concentrations. Hydén made his measurements on conscious sheep, using polyethylene glycol as a marker. His observations on sodium and potassium absorption from the rumen are similar to Dobson's, and he also showed that virtually no phosphate is absorbed.

There is no significant net movement of calcium and magnesium through the rumen epithelium (see Rook & Storry, 1962) whose potential gradient will strongly oppose the absorption of divalent cations. The absorption of fatty acids and bicarbonate have been considered in the previous section.

Sodium and potassium together account for most of the cations present in the rumen fluid and their concentrations show a strong inverse correlation (Bailey, 1961*b*). Dobson and co-workers (Sellers & Dobson, 1960; Dobson & McDonald, 1963; Dobson, 1963) have studied the relationships of rumen sodium and potassium concentrations, rumen potentials and diet. Around 90–100 m-equiv. of Na^+/l and 30–40 m-equiv. of K^+/l were found in the rumen on diets of hay or hay and meals. These concentrations were reversed and the rumen potential tended to rise when grass containing a lot of potassium and very little sodium was fed. There is no obvious explanation for the fall in the sodium : potassium ratio in the rumen. It happened so swiftly and surely that it cannot have been due to the slower and inconstant fall in the sodium : potassium ratio in the saliva that sometimes occurred. So little sodium was eaten with any of the diets that it can have had little direct effect on its concentration in the rumen. Possibly the tendency for isotonicity to be preserved when large amounts of potassium are released from the food prevents the absorption of water from the rumen and so reduces the concentration of sodium. The rumen sodium : potassium ratio began to rise slowly after a week or so on the grass diets although there was little or no corresponding change in the composition of the grass itself.

The relative importance of saliva and of food in controlling electrolyte concentrations in the rumen has also been studied by Bailey (1961*b*). Samples of mixed saliva and rumen fluid were taken from cattle at intervals after feeding on a variety of diets. The concentration of sodium in the rumen was proportional to but a little less than

its concentration in the saliva, and this emphasizes the overwhelming importance of salivary sodium. The concentrations of potassium and chloride in the rumen fluid were higher than in the saliva. The potassium concentrations in the two fluids were correlated but the chloride concentrations were not. The concentrations of both ions in the rumen rose after feeding and probably their most important source was the food. The concentration of inorganic phosphate in the rumen ranged from 3 to 45 m-equiv. of HPO_4^{2-}/l and was not related to either dietary or salivary phosphate levels.

McClymont, Wynne, Briggs & Franklin (1957) found that wethers fattening on diets of oats or oats with lucerne or wheaten chaff, which supplied only 4–38 m-equiv. of sodium daily, ate more and grew faster when NaCl was added to the diet. Dry ewes on maintenance rations of oats, on the other hand, seemed to derive no benefit at all from a salt supplement (Briggs, Franklin & McClymont, 1957). The extraordinary indifference of non-productive sheep to sodium scarcity may be due partly to the negligible loss of sodium in sweat and partly to the efficiency with which they can conserve sodium. In the author's experience (unpublished results) the urinary and faecal losses of sodium may total only 2–5 m-equiv. daily on low-sodium diets or during sodium depletion. The rumen fluid normally contains several hundred m-equiv. of sodium. When sheep are depleted of sodium, potassium largely replaces sodium in the parotid saliva and a similar change takes place in the composition of the rumen fluid so that the sheep withdraws sodium from its gut into its body fluids (Denton, 1957*a*; Denton *et al.* 1961). The rumen fluid therefore serves as a reservoir for sodium which can be drawn upon to tide over periods of seasonal dietary deficiency or to meet the needs of pregnancy, etc., and is re-stocked when sodium is more freely available.

A considerable amount of work has been done to find whether supplements of minerals or ash improve appetite or digestion and animal productivity. Barnett & Reid (1961) devote a chapter to the part played by minerals in the rumen. Some more recent work is referred to in papers by Nicholson *et al.* (1962). All that is required here are a few cautionary remarks. The concentration at which various minerals have been shown to stimulate the activity of rumen cultures *in vitro* is often considerably below the normal rumen range, so that no such effect need be expected by using mineral supplements *in vivo*. Potassium, for example, stimulates cellulolysis *in vitro* at concentrations of 500 μg/ml (13 m-equiv./l) and over (Hubbert, Cheng & Burroughs, 1958), but in fact potassium concentrations in the rumen are usually well above this level. Phosphorus in various forms has a similar effect *in vitro* at concentrations of 20 μg/ml (1·3 m-equiv. HPO_4^{2-}/l) and over (Hall, Baxter & Hobbs, 1961) but again rumen levels are much higher.

In some feeding trials supplements of alfalfa ash or of salts such as NaCl, $NaHCO_3$, $KHCO_3$, $CaCO_3$, sodium acetate or citrate, etc., have been shown to improve appetite or feed conversion efficiency and growth, while other trials have given negative results. When salt supplements have improved animal performance it is often difficult or impossible to decide from the results reported whether the supplement acted by repairing a deficiency, by supplying buffer to an over-acid rumen, by increasing water intake and so the fluidity and outflow of the rumen contents, or in some other way. In a sobering experiment Hix, Evans & Underbjerg (1953) have shown that the additional weight gained by lambs when they are given a salt supplement can

be due entirely to retention of water and salt as extracellular fluid. Research into the effects of salt supplements is likely to remain confused and unpredictable until a more complete understanding is gained of the part played by salts in the digestive physiology of ruminants. A useful step in this direction has been taken by Nicholson *et al.* (1960) who fed a roughage diet containing little ash to groups of calves. Some groups were given supplements of alfalfa ash or alkaline minerals and animal performance was related to various characteristics of the rumen environment. The supplements caused an increase in food intake and growth and were associated with increases in the percentage of water in the rumen contents, in the rate of cellulolysis and in urine pH. The osmotic pressure and pH of the rumen contents depended primarily on the concentration of volatile fatty acids.

RE-USE OF UREA NITROGEN

Most dietary nitrogen is degraded in the rumen to form ammonia. Part of this is then used in the synthesis of microbial protein, which accounts for most of the nitrogen passing to the omasum (Weller *et al.* 1962). The microbial reactions centred on the rumen ammonia pool are described in Part 2 (p. 298).

Direct measurements of the amount of nitrogen in the digesta leaving the omasum and entering the duodenum in conscious animals have enabled reliable estimates to be made of the net amounts of nitrogen lost or gained by food in its passage through the stomach. This may also be calculated from analysis of gut contents in slaughtered animals, although the rapidity with which the intestinal epithelium sloughs off after death (Badawy, Campbell, Cuthbertson & Fell, 1957) makes such calculations rather suspect. Net losses of nitrogen from the stomach contents occur when the amount of nitrogen in the diet is high (Badawy, Campbell, Cuthbertson, Fell & Mackie, 1958; Kameoka & Morimoto, 1959; Hogan & Phillipson, 1960; Ridges & Singleton, 1962). The greatest losses occur when little digestible carbohydrate is available to encourage microbial protein synthesis (Phillipson, Dobson, Blackburn & Brown, 1962) or when the dietary protein is particularly soluble (Tagari, Ascarelli & Bondi, 1962). Under these conditions the ammonia concentration in the rumen rises so that a considerable amount of ammonia is absorbed into the portal blood and converted to urea by the liver (Lewis, Hill & Annison, 1957; McDonald, 1958).

On the other hand, when the nitrogen intake is low there may be a net addition of nitrogen to the stomach contents, sometimes amounting to 4–5 g daily in sheep (Gray, Pilgrim & Weller, 1958; Harris & Phillipson, 1962; Kay & Phillipson, 1962). Little ammonia is absorbed from the rumen (Lewis *et al.* 1957) and dietary supplements of urea are efficiently utilized (Balch & Campling, 1961). Kameoka & Morimoto (1959, Trial 11) put a 22 kg goat on to a nitrogen-free diet, supplemented by casein given into the abomasum, and from their report it can be estimated that about 2 g of endogenous nitrogen were added to the fore-stomach daily. Probably most of the nitrogen gained by the rumen contents passes into the rumen as urea. Nitrogen may also be added to the rumen in significant quantities as salivary protein (Somers, 1961), the secretions of the respiratory tract and desquamated epithelium. There is also the possibility, as Hobson (1959) has pointed out, that gaseous nitrogen may be fixed anaerobically in the rumen. Nitrogen fixation, possible or proven, by micro-

organisms, plants and animals is discussed by McKee (1962, Chapter 3). Nitrogen is also secreted into the abomasum as urea (Le Bars & Simonnet, 1959) and mucoprotein. The urea secreted into the rumen is rapidly hydrolysed by bacterial urease and adds to the ammonia pool, and on low nitrogen diets this additional supply of ammonia helps to promote an active microbial population in the rumen and increases the synthesis of microbial protein (Moir & Harris, 1962).

The ruminants are therefore able to re-use large amounts of urea nitrogen which otherwise would be lost in the urine and this seems to be an important adaptation to the inadequacies of their diet. This cycle is made still more effective by a renal mechanism that allows urea to be retained in the blood. Schmidt-Nielsen, Schmidt-Nielsen, Houpt & Jarnum (1957) found that camels excreted very little urea in their urine when a diet low in nitrogen was fed, especially if the animals were also dehydrated. When urea was injected intravenously little was lost in the urine and the authors concluded that it must be passing into the rumen instead. Further experiments showed that the kidneys of sheep and man can also conserve urea when the nitrogen intake is low. The latest paper is that by Schmidt-Nielsen & O'Dell (1959). When sheep are given a diet that contains only 2–3 % of digestible protein or less, the concentration of urea in the urine falls so that it does not exceed ten times the concentration in the blood. The glomerular filtration rate is unaltered and the extent to which urea is concentrated is probably limited by active reabsorption of urea by the kidney tubules. The stimulus to this reabsorption is associated with the low nitrogen intake itself, and not with the concentration of urea in the blood, since intravenous infusions of urea given to sheep on a low nitrogen intake are conserved about as efficiently as endogenous urea. The urea-conserving mechanism cannot operate at high urine flows, since the concentration of urea in the urine then does not approach ten times the blood level. The practical importance of this last fact is shown clearly by the experiments of Livingston, Payne & Friend (1962). Zebu and European steers were given food with a crude protein content which was reduced successively from 12 to 8 and finally to 4 %. With water freely available the urinary excretion of urea fell, but only in rough proportion to the nitrogen content of the ration; presumably the urine flow was too great to permit active retention of urea. However, when, on the 4 % protein diet, the water intake was severely restricted the urine flow dropped and urea excretion dwindled to only a fifth of its previous rate, allowing the conservation of about 12 g of nitrogen daily.

Urea may pass from the blood to the rumen contents either in the saliva or directly through the rumen epithelium. The potential importance of the salivary route has long been recognized and recently the secretion of nitrogen in the saliva of sheep has been examined carefully and quantitatively by Somers (1961). Phillipson & Mangan (1959) had earlier shown that parotid, submaxillary and mixed salivary secretions contain similar concentrations of urea. Somers found that this concentration was about a half to three-quarters of the blood urea concentration, as it is in cattle (Bailey & Balch, 1961*b*), and that parotid urea increased in parallel with blood urea when intracarotid injections of urea were given or the dietary nitrogen intake was raised. Parotid urea also rose when the rate of secretion was increased. Parotid and blood urea concentrations and rumen ammonia were all raised after feeding. The total amount of nitrogen returned to the rumen by one parotid gland was

equivalent to nearly 2% of the dietary nitrogen intake, whether the intake were 14·5 or 6·8 g of nitrogen daily. Urea nitrogen accounted for about half of the salivary nitrogen on the high nitrogen intake and nearly three-quarters on the low intake. Since the saliva secreted by one parotid is probably little more than a quarter of the total salivary secretion (Kay, 1960*a*) and the total nitrogen content of mixed saliva is rather greater than that of parotid saliva, the total amount of nitrogen secreted in the saliva is probably roughly one-tenth of that in the diet. Further experiments by Somers showed that, as a result of renal nitrogen retention, the recycling of urea became more efficient when the nitrogen intake was low, even though his sheep had free access to water. An infusion of 1·4 g of urea nitrogen was given daily into a carotid artery. When the sheep were in positive nitrogen balance 1·0 g of this dose was recovered in the urine and 0·1 g in the additional salivary urea secreted by one parotid gland; when the sheep were in negative balance only 0·3 g was recovered in the urine and a further 0·3 g in the saliva. Houpt (1959) showed that the retention of injected urea was increased when a carbohydrate-rich diet was fed.

Houpt (1959) also studied the rate at which urea passed directly into the rumen contents by diffusion through the rumen epithelium. Ammonia accumulated in saline solutions placed in the isolated rumen of anaesthetized sheep at a rate of about 2·5 m-moles/h; this was the result of bacterial hydrolysis of the urea that diffused in from the blood. When allowance was made for the amount of ammonia that was absorbed from the rumen, which was calculated rather circuitously, the rate of appearance of ammonia was roughly doubled and so became equivalent to the passage of about 1·7 g of urea nitrogen daily into the rumen contents. Le Bars & Simonnet (1959) and Ash & Dobson (1963) reported rather slower movements of urea through the rumen epithelium. All these rates will be on the low side if the passage of urea through the rumen epithelium is depressed by anaesthesia and surgical interference.

None of the rumen workers reported the nitrogen intake of their sheep so that one cannot yet reliably compare the amounts of urea passing through the rumen epithelium with those returned to the rumen in the saliva. But present indications are, as Houpt (1959) and Moir & Harris (1962) have suggested, that the rumen epithelium will prove the more important route.

PART 2. RUMEN MICROBIOLOGY

INTRODUCTION

In the last few years many papers have been published which deal with some aspect of ruminant digestion. It would be impossible in one short review to discuss all the many problems that are covered by these papers, some selection therefore being necessary. In the field of rumen microbiology there have also been a number of recent reviews. The monographs of Annison & Lewis (1959) and Barnett & Reid (1961) each has a portion devoted to microbiology. Cuthbertson & Hobson (1961) described the role of micro-organisms in the conversion of the constituents of feedstuffs to products which can be utilized by the host, and the data then known of the types of bacteria and protozoa responsible for these reactions were given. The reader is referred to that review for an overall picture of the fate of feedstuff constituents in

the rumen and for earlier references not mentioned here. Another review by Bryant (1959) has described the properties of many strains of the most important rumen bacteria, and Hungate (1960) reviewed some aspects of rumen microbial ecology. It seemed to the author that no good purpose would be served by recounting much of the material given in these reviews and so three general topics have been chosen in which significant advances have been made in the last 2 or 3 years. Some of the latest work on the identification of the types of organisms responsible for breakdown of certain feedstuffs will be given, as this information was not available to the earlier reviewers. Most emphasis will be placed on recent work which shows the importance of some of the products of microbial synthesis, the metabolic pathways of the rumen organisms which fit them for life in the rumen, how this metabolism is of importance to the host, and some of the factors influencing the rumen microbial population. Although recent studies, especially with pure cultures of rumen bacteria, have added greatly to our knowledge of the details, the general outline of many of the results discussed here has been known for some years. For this reason it has not been thought necessary to give a reference to support every general statement made, and because of space limitations only one reference for a topic has been given in some cases. This reference has, I hope, been one of the latest and one which gives other references to earlier work, from which a more detailed picture of the subject can be built up.

THE DEVELOPMENT OF THE RUMEN MICROBIAL POPULATION IN YOUNG ANIMALS

The physiological development of the rumen in young animals was dealt with in the first part where it was shown that the maturation of the rumen and the salivary glands depends more on the intake of solid food and the establishment of ruminal fermentation than on the age of the animal. Since the metabolic products of the rumen bacteria are important it would seem that functioning rumen bacteria are the first steps towards a fully functioning rumen. However, the rumen microbial population depends on the feed consumed and it is impossible to generalize about the relationship of age and microflora. What is probably important is the change from a predominantly lactic acid-producing flora in the young animal to a more varied population, producing a larger proportion of volatile fatty acids, as solid feed is consumed. Since many types of adult-rumen bacteria produce mainly fatty acids and the metabolic products of the protozoa are similar, the actual types of bacteria or the presence or absence of protozoa may not be so important for development of the rumen. Whilst it may not be possible to feed a ruminant from birth on a solid ration the practice now is tending towards earlier weaning and replacement of milk by a concentrate-containing feed. The rumen population of the very young animal is most likely derived from backflow of the contents of the abomasum and contains large numbers of lactobacilli, streptococci and coliforms (Mann & Oxford, 1955; Mackay & Oxford, 1954). Bryant, Small, Bouma & Robinson (1958*a*) found large numbers of lactic acid bacteria in calves from 1 to 6 weeks of age fed on milk, but with access to hay and concentrates, and the presence of these organisms may be ascribed to the milk. Lactate-utilizing bacteria are also found in high numbers in young animals (Bryant *et al.* 1958*a*; Hobson, Mann & Oxford, 1958). Although

cellulolytic bacteria were found in the rumens of these calves (Bryant *et al.* 1958*a*) from 1 week old the predominant bacteria from the calves at 1–3 weeks were different from those of mature animals. For instance, the numbers of facultative anaerobes were high in the calves. By 6 weeks many groups were the same as in mature animals, but several groups not found in mature animals were still present. At 13 weeks of age the rumen bacteria were predominantly those of mature animals. The changes in the bacterial populations were paralleled by changes in the diet. Although milk was fed during the experiment the voluntary consumption of hay and grain increased rapidly, especially after the sixth week. The calves did not develop a ciliate protozoa population during their first 13 weeks of life, probably because they were not kept near to mature cattle from which inocula of protozoa might have been obtained. The effect of diet on the bacterial and protozoal population of young animals is illustrated by the work of Eadie, Hobson & Mann (1959), where calves were fed milk alone to 1 week old and weaned at 21 days on to diets of preponderantly concentrates or a 3:1 mixture of dried grass and concentrates. In the animal on the concentrate diet the high numbers of lactobacilli and the lactate-utilizing *Peptostreptococcus elsdenii* established at 2 weeks old persisted for about 15 weeks then slowly declined, whereas in the calf getting a higher proportion of dried grass the initially high numbers of lactobacilli and *P. elsdenii* fell rapidly after weaning, and remained low until at 16 weeks old the concentrates were increased and the grass reduced when the numbers of these organisms rapidly rose to the levels of the other animal. Each animal was regularly inoculated with mixed rumen protozoa and a mixed fauna was established and persisted in the calf getting grass and concentrates, whereas no protozoa were seen in the concentrate-fed calf until it was 12 weeks old and they did not reach the level found in the other calf until after another 5 weeks. The establishment of rumen protozoa appears to depend upon inoculation from either a neighbouring adult animal or some chance source, and upon the pH of the rumen contents, which is influenced by the types of rumen bacteria and the diet of the animal. Purser & Moir (1959) noted that a low pH inhibited the multiplication of protozoa in adult sheep, and in the observations of Eadie, Hobson & Mann (1959) a correlation between a low rumen pH, high lactobacillus counts, and lack of rumen protozoa was noted. Eadie (1962*a*) has examined in more detail the establishment of protozoa in young animals kept under different conditions of management. She found that, although there were variations between individual animals of the same species and some differences between calves and lambs, diet, not age, was the governing factor in development of a ciliate population. Ciliates could be established in lambs as early as 9 days old if the rumen pH, which was governed by the diet, was favourable. If the rumen pH was below 6·0, as was obtained on feeding a high concentrate ration, then a ciliate population was not established. Or, if ciliates had been established, change of the ration to a high-concentrate basis caused their disappearance. If the rumen pH was a little above 6·0 entodinia were the predominant ciliates, and if the pH was above 6·5 a mixed ciliate population developed. In animals weaned on to certain mixtures of hay and concentrates the rumen pH remained high and ciliate populations could be established and maintained. Young animals kept in isolation can be reared and kept indefinitely without protozoa, and to compensate for their absence the ruminal bacterial population increases (Eadie & Hobson, 1962).

Establishment of protozoa depends, except in the case of some very small protozoa which can be carried in airborne droplets (Eadie, 1962*a*), on close contact between the young animals and adult faunated animals. However, unless young animals are very well isolated they will develop normal rumen bacteria through feed or airborne contamination. Mann (unpublished observations) has found in the air of cattle sheds small numbers of viable, strictly anaerobic bacteria which on preliminary examination resemble types found in the rumen. The value of rumen inoculation, as an aid to development of adult rumen bacteria and increased ability to digest roughages appears to be doubtful under normal conditions. Work on the subject of calf performance and rumen inoculations is reported in papers by Pounden & Hibbs (1948); Conrad & Hibbs (1953); Hibbs & Conrad (1958); Hardison, Miller & Graf (1957); McArthur (1957); Preston (1958); Pelissier, Slack, Trimberger, Turk & Loosli (1954); Ackerman & Fike (1955). Bryant & Small (1960) reported studies on the bacteria of inoculated calves. If the diet of the animal is suitable it will develop the correct bacterial population. Whether the inoculum proliferates is dependent on the suitability of the diet. In addition to this it seems doubtful whether any commercially available rumen inocula, at any rate, contain any viable rumen organisms.

However, once the rumen has developed and the animal has adjusted itself to life on an adult diet the rumen becomes a comparatively stable system. The adult rumen population is considered in the next section.

VARIABILITY AND STABILITY OF THE RUMEN FLORA AND FAUNA

Introduction

Although the rumen is a remarkably constant environment compared with the habitats of many micro-organisms, the flora is subjected to some variation in conditions. Amongst the factors which will influence the organisms are the secretions of the host animal. The saliva will exert an influence by its volume, which will affect the dilution rate of the rumen culture, its buffering capacity, its ionic content, and its surface tension. The secretion into the rumen of urea and salts from saliva or via the rumen epithelium will influence microbial activity. Rates of absorption of microbial fermentation products will also influence the microbial growth. The physical state, type and rate of administration of feed are other variables. The type of feed will exert influence not only by its gross composition in terms of carbohydrate and protein but by its content of trace elements and other minor constituents which are needed for optimum growth of micro-organisms. Some of these factors may be outside the control of the host animal, others are probably influenced by feed-back mechanisms, or mass action effects initiated by the microbial metabolism and generally operating in such a way as to stabilize the rumen at the optimum conditions for feed digestion. The physiological mechanisms that influence the rumen environment have been reviewed in the first part of this article. In the following section some recent observations on stability and variations in the rumen microbial population are described.

It would seem that in general observations in rumen microbiology can be applied equally to sheep and cattle, and probably to ruminants in general. Which of the two main domesticated species of ruminant has been used for observations has depended

largely on outside influences, such as availability and ease of handling of animals. Where a difference between host species has been found reference has been made to the fact.

Factors influencing the rumen population

Most of the *in vivo* work on the composition of rumen ingesta has been done on animals fed once or twice a day, and under these conditions there are likely to be large fluctuations in microbial nutrients and metabolic products and in the numbers and activity of the micro-organisms. These conditions will apply to stall-fed animals, but in the grazing animal there will tend to be a more constant supply of nutrients for the rumen organisms and their metabolic products will be released at a more constant rate. Most of the rumen organisms show a great versatility in the substrates which they will attack and it would seem probable that any one type of organism will be utilizing different nutrients at different times depending on whether feed has been recently ingested by the host. Fluctuations in the available nutrients will be affected not only by frequency of feeding, but also by the type of substrate. Thus whilst soluble carbohydrate and nitrogenous materials are very rapidly broken down (for proteins, see e.g. Blackburn & Hobson, 1960*a*; Moore & King, 1958) more resistant forms of carbohydrate and protein are digested, and their hydrolysis products liberated at a slower rate and this will lead to a more uniform metabolism of the rumen organisms. Also the composition of the feed will affect the rate of its passage through the rumen, the rate of salivary flow, and hence the rate of digestion of the feed. The rumen may, therefore, be likened to a kind of continuous culture of mixed micro-organisms with bursts of activity of one or more types of organisms depending on the inflow of feed, combined with a sort of basal metabolism which keeps the numbers of organisms approximately constant. This 'basal metabolism' might be a fermentation of substrates slowly released by the disintegration of more resistant feed particles or dead micro-organisms, or endogenous metabolism of reserve starch-type polysaccharides or other cell constituents. (The formation and utilization of starch-type polysaccharide has been noted in a pure culture of a rumen bacterium by Doetsch, Howard, Mann & Oxford (1957) and in mixed rumen bacteria *in vitro* by a number of workers, e.g. Gibbons, Doetsch & Shaw (1955).) However, the theories developed for the description of *in vitro* continuous culture (Monod, 1950; Herbert, Elsworth & Telling, 1956) cannot strictly be applied to the rumen environment as the culture is made up of many types of organisms, the rate of addition of nutrients is not constant, the limiting nutrient for growth of the organisms must be changing, and in addition the same nutrient will not be limiting for all organisms at any one time. Also not all the organisms are free in a liquid environment, many are attached to plant particles and their rate of growth may be governed both by the supply of nutrient in the fluid around the solid and the availability of some constituent of the plant particle. Whether the bacteria are fixed or free will probably be reflected in their rate of passage out of the rumen. However, although there are fluctuations in numbers of rumen organisms, once stabilized to a particular diet of the host animal the rumen 'culture' is very resistant to change from external contamination. For instance, large numbers of sporing bacteria and other organisms which can grow under the anaerobic conditions used in *in vitro* culture of rumen bacteria must be

ingested with the feed of the host animal, but the numbers of viable bacteria of these kinds found in the rumen under normal conditions are low. As examples of this Appleby (1955) estimated that her experimental sheep would be ingesting 10^5–10^6 bacilli and spores/g of hay eaten, but bacilli are not usually found in large numbers in the rumen. Gutierrez (1953) found propionibacteria in the rumens of some cattle being fed on hay in average numbers of 10^6/ml. However, the hay itself contained 10^8 or 10^9 propionibacteria/g and although the bacteria were growing in the rumen, it seemed doubtful if this was the site of active growth. The numbers were probably being kept up, but only to a comparatively low level, by continuous inoculation from the hay. It may be that in the *in vivo* rumen environment the growth rate of these bacteria is lower than the rate of flow through the rumen, so that they are 'washed out', or there may be other factors inhibiting their growth. Hungate (1960) suggested that the facultative anaerobic bacteria may be handicapped by carrying the mechanism for formation of enzymes of aerobic and anaerobic metabolism, the former being wasted in the rumen environment. However, apart from 'foreign' organisms introduced with the feed which may be unsuited for life in the rumen it is also difficult to establish organisms which will flourish in the rumen at other times. For instance, the large Gram-negative coccus, *Peptostreptococcus elsdenii* (Elsden, Volcani, Gilchrist & Lewis, 1956; Gutierrez, Davis, Lindahl & Warwick, 1959) is one of the predominant bacteria in the rumens of young calves (10^9–10^{10}/ml) but is found only in low numbers (about 10^4/ml) in older animals and sheep (Hobson *et al.* 1958). Hobson & Mann (1961*a*) attempted, by massive inoculations of *P. elsdenii* which had been grown in pure culture *in vitro*, to influence the numbers of this organism in the sheep rumen and in older calves in which the numbers of *P. elsdenii* had decreased to 10^3–10^4/ml. Although the numbers of *P. elsdenii* were for a short time brought up to 10^8/ml, within 1 or 2 h the numbers had fallen drastically and within about 6 h were down to the pre-inoculation level. This pattern of events was followed even after repeated inoculations and in animals on different diets, some of which were similar to those of young animals in which the organism flourished. The addition to the diet of sodium lactate, which is a good substrate for growth of the bacteria *in vitro*, or inoculation of an older calf with large amounts of rumen contents from a younger animal which contained *P. elsdenii* and the bacteria associated with it, also failed to produce a stable large population of these bacteria. Thus the lack of growth of the bacteria may be due to competition for limited substrates from the established population, to ingestion by protozoa of the adult population, or to the presence in large amounts in the flora of the young rumen of growth factors which are not produced by the micro-organisms of older animals, or there may be some factor due to the animal itself which varies with age. The only reported incidence of these bacteria in large numbers in adult animals is in steers suffering from feed-lot bloat (Gutierrez *et al.* 1959), and here the diet may play some part, by introducing starchy materials like those in the diet of the young animal and altering the whole balance of the adult flora and fauna. That the diet has an effect on the numbers of bacteria in young animals has already been mentioned, but that diet is not the only factor influencing the growth of bacteria in the adult rumen is shown by the experiments noted above and by the fact that *P. elsdenii* disappears from the rumen of calves as they grow older even though their diet is changing only in amount eaten and not in

composition. That similar difficulties can be found on endeavouring to change the rumen protozoal population is shown by some experiments of Eadie (1962*b*). In this work an apparent antagonism between some of the large oligotrich protozoa was found, and a stable population containing both *Polyplastron multivesiculatum* and *Eudiplodinium maggii* or *Epidinium* spp. could not be obtained. In sheep it seemed that the dominant population was one containing *P. multivesiculatum*, and although a population of the other types could become established, natural or artificial inoculation with a comparatively few *P. multivesiculatum* resulted in a change of population which could not be reversed by repeated inoculation with *Eudiplodinium* and *Epidinium*. There appeared to be some animal effect in this case as although the polyplastron population was dominant in sheep, in some calves the *Eudiplodinium-Epidinium* population appeared also to be stable and to resist change on addition of polyplastron.

However, when the conditions for growth of the organisms are correct a very small inoculum, even such as could be carried in droplets in the air, must suffice to introduce bacteria and protozoa into the rumen, as the rumen population of growing animals changes due to contamination from neighbouring animals.

That an established rumen population is in a state of dynamic equilibrium can be shown by experiments such as those of Hobson *et al.* (1958) where the serological types of *P. elsdenii* were followed in two calves in which the total numbers of these bacteria remained constant at a high level over a long period. During this time the serological types fluctuated markedly. It could not be established whether this was due to mutations occurring or to natural re-inoculation by different serological types, but an attempt to change the serological type by artificial inoculation was unsuccessful. The protozoal and bacterial populations are in equilibrium as well. Eadie & Hobson (1962) showed that the rumen bacterial population in lambs, kept without protozoa for periods of more than 2 years, was consistently higher than in animals on a similar diet with a mixed bacterial and protozoal population. When protozoa were established in the previously unfaunated lambs the bacterial population fell to the usual equilibrium level. Here competition for nutrients between bacteria and protozoa may be the main factor, although a possible contribution could be made by the ingestion of bacteria by the protozoa (Gutierrez, 1958; Gutierrez & Davis, 1959). It is somewhat difficult to decide how the total numbers of rumen bacteria and protozoa change on changing the type of feed of the host animal. There are differences in the relative proportions of micro-organisms with differences in diet and these can sometimes be seen by qualitative observations (for instance, entodinia often predominate amongst the protozoa in an animal ingesting a starchy concentrate ration). A difference in the relative numbers may not, however, be shown in a difference in microscopic count, especially if the relative proportions of small, morphologically similar, bacteria are changing. The difficulties in assessing changes in numbers from published data lie to some extent in the differences between total and viable counts of bacteria, and to the fact that protozoal and bacterial counts have rarely been made on the same animal, largely owing to technical difficulties. Also the relative accuracies of viable and total counts vary with the methods used. Other complicating factors are diurnal and seasonal variations in numbers of organisms and variations between animals on the same diet, or between the numbers in the same animal on different

days (see, for instance, Nottle, 1956; Eadie & Hobson, 1962). This latter point was especially marked in the viable counts of bacteria in a concentrate-fed animal made by Bryant & Robinson (1961*a*); the explanation is at present unknown. Warner (1962) made detailed total counts of rumen micro-organisms in a number of sheep. He found that there was an increase in numbers of all the morphological types of organisms counted when the ration of one sheep was changed from hay (900 g/day) to hay (300 g/day) plus groundnut meal (300 g/day) plus flaked maize (300 g/day), and that the change appeared complete in about 10 days. However, he also found that there were very large variations in numbers of organisms between animals on the same ration and, as others have found, that there were diurnal variations in total numbers (the animals were fed once a day). The pattern of diurnal variation varied with the type of organism, which might be expected if the organisms were utilizing different components of the diet. (Purser & Moir (1959) and Purser (1961) have also observed differences in the diurnal cycle of holotrich and oligotrich protozoa.) The different species of organisms also showed differences in their fluctuations in numbers over the period of the experiments. Moir & Somers (1957) found that the pattern of diurnal variations in total numbers of bacteria in the rumens of sheep varied with the method of feeding, i.e. whether a ration was given as one or more feeds per day. However, they found that the average concentrations of bacteria did not vary with method of feeding and did not vary between animals, contrary to the results quoted above, but the numbers of protozoa did vary with method of feeding. If the flow from the rumen is not constant, though, average numbers of bacteria may bear little relationship to the microbial activity.

Total counts may not give a true idea of the extent of microbial activity at any one time, although they must bear a relationship to the average microbial activity over a period, as total and viable counts of bacteria usually differ markedly. The viable count of bacteria, especially in animals on a hay ration, is not more than a few per cent of the total count; in animals on a concentrate ration it is often higher, but still not near the total. This may be due to deficiencies in the media used for viable counts or it may mean that a large proportion of the bacteria is dead, and that the number is limited by the exhaustion of nutrients or by other factors such as the accumulation of end products. However, bacteria which are dead in the sense that they will not reproduce in a suitable medium may still be capable of accumulating products such as intracellular polysaccharides (Hobson & Mann, 1955), and bacteria limited in growth by lack of nitrogen may still continue to ferment carbohydrates. Ingested food may increase the rate of fermentation, but may not increase the microbial numbers (Walter, 1952). Some results suggest that the numbers of bacteria are limited by lack of a particular nutrient. The total counts of Moir & Williams (1950) suggested that nitrogen was the limiting factor for bacterial numbers in sheep fed oat hay, starch and increasing quantities of casein. The total numbers of bacteria showed a linear relationship with the protein content of the feed. Nitrogen may also have been limiting the viable bacteria in the experiments of Krogh described below and much of the excess sugar could have been fermented by non-proliferating bacteria. The form in which nitrogen is fed can also influence the total numbers of bacteria in the rumen (see, for instance, Williams & Moir, 1951).

Warner (1962) pointed out that the fluctuations in numbers and types of micro-

organisms with time or between animals were apparently much greater than the fluctuations noted by measurements of the chemical activities in the rumen (ammonia concentration, nitrogen balance, etc.). This could be ascribed to the fact that the end products of fermentation of carbohydrates by a number of types of organisms are similar and also that an increase in, say, numbers of bacteria forming rumen intermediates, such as lactic acid, could be balanced by an increase in bacteria fermenting it. Bauchop & Elsden (1960) and Sokatch & Gunsalus (1957) have shown that the cell yield from fermentation of a certain weight of a sugar can differ in different bacteria according to the pathway of metabolism if this sugar is a limiting nutrient, and so different bacterial numbers in the rumen might not indicate different yields of fermentation products.

However, recent work by Gilchrist & Kistner (1962) suggests that on some diets of constant composition, but decreasing quantity, the concentrations and types of viable rumen bacteria can remain constant even though the diet may be quite inadequate to maintain the sheep at constant weight. The rate of multiplication of the bacteria on the inadequate diets may, however, be slowed down, for the volume of saliva secreted will fall in proportion to the amount of dry matter eaten and so tend to maintain the bacterial concentration. This hypothesis would only apply, however, if the concentration of nutrients was still adequate to support the bacteria even on the smallest feed intake. It is also possible that a smaller amount of nutrients is needed to maintain the rumen microbial population than is required to maintain adequately the host animal. However, as the animals in these experiments were accustomed to a diet of poor hay the numbers and types of bacteria initially present may have been smaller than in animals on a high plane of nutrition, so that the results might not apply on decreasing the quantity of a high plane diet. That the concentration of carbohydrate in the diet may not be the factor limiting the microbial numbers is suggested by the work of Krogh (1959, 1960, 1961) who added increasing quantities of carbohydrate to the diet of sheep. Viable counts of different types of bacteria showed little change in numbers until a very high level of carbohydrate was being fed (300–400 g of lactose, 200–300 g of sucrose or 1500 g of starch per day), when a drastic change in the flora took place, the normal flora being replaced by a preponderantly Gram-positive one, this also being accompanied by severe illness in the animal. Whether or not the rumen population is changed by a change in diet thus depends on a number of factors. If additional carbohydrate and utilizable nitrogen are given to a diet low in these constituents, then an increase in number of organisms will probably take place. However, an increase in carbohydrate or nitrogen alone will not lead to an increase in numbers if the nitrogen or energy supply is inadequate. The concentrations of trace elements, sulphur, and other growth factors could also limit changes in the microbial population on changing the diet.

Regeneration of the rumen population

Fluctuations in numbers of particular organisms which appear random may mean that some organisms tend to die off and are re-introduced by inoculation from outside the animal. The rumen population shows a remarkable tendency to return to normal through re-inoculation or regrowth of residual organisms even after drastic alteration. In the experiments of Krogh described above, when the rumen population had been

drastically altered by feeding carbohydrate, change to a normal diet resulted in return to normal of the rumen flora in about 2 days. Warner (1962) found that the smaller bacteria returned to normal numbers within a few days after being decreased in numbers by starvation of the host for 4 days, and the larger organisms also usually reappeared within a few days, although the times of reappearance varied in different experiments. The results suggested that for most organisms repopulation was due to growth of the small residuum of organisms in the rumen of the starving sheep, although in some cases repopulation may have been due to re-inoculation from external sources. Meiske, Salsbury, Hoefer & Luecke (1958) found that *in vitro* cellulolysis by mixed rumen organisms was decreased by 68–91 % on starving the donor steer for 3 days. Cellulolytic activity returned to normal 3–6 days after feeding started again. Other workers have found similar rapid recovery in metabolic activity of rumen contents.

SOME ASPECTS OF RUMEN MICROBIAL METABOLISM

Experimental methods

Since the rumen population is a kind of continuous culture of different micro-organisms, many of which may be dependent on the action of others for a supply of nutrients, it is often difficult to relate experiments *in vitro* in an entirely quantitative and satisfactory manner to the processes *in vivo*. *In vitro* experiments with pure cultures are usually done on a batch basis and it is known that rate of growth and also concentration of substrate and composition of medium can affect not only the composition of the bacterial cells but also the fermentation products. Washed suspension experiments in different forms are used in studies of rumen metabolism, but here again one is using a system differing markedly from the proliferating rumen culture. Hueter, Gibbons, Shaw & Doetsch (1958) have compared *in vitro* washed suspension and *in vivo* experiments and have concluded that the washed cell suspension technique appears most useful for studying short one- or two-step reactions presumed to occur in the rumen. The technique loses significance when studying multi-step metabolic reactions and in such cases the results should be interpreted with caution. A criticism of the 'artificial rumen' type of experiment is that in most cases rumen contents are treated in some way, such as centrifuging, before being used in the experiments and where experiments are prolonged the final microbial population may not be that which was initially obtained from the rumen. Consequently the rates of fermentation, the concentration of products, and the materials fermented may change (see, for instance, Rice, Salsbury, Hoefer & Luecke (1962), who also give references to earlier workers). Some designs of artificial rumens have been produced which endeavour to simulate rumen action in a number of respects and to keep the microbial population in the same balance as that of the original rumen fluid. However, most of these seem to run for only a few hours or a day before changes in the microbial population and activity take place or mechanical failures occur (Adler, Dye, Boggs & Williams, 1958; Stewart, Warner & Seeley, 1961). The one published design of artificial rumen which has been run for up to 3 weeks and which appears to simulate rumen function over this time is that of Davey, Cheeseman & Briggs (1960), although here again the published data do not make it certain that

the population of the artificial rumen after a prolonged experiment is the same as that of the natural rumen. It would seem that further useful work on rumen metabolic pathways could be carried out on the lines of the 'continuous' cultures of whole rumen contents which have been pioneered by a number of workers. However, it is impossible to lay down rules for the study of rumen function, for different aspects of the problem require different approaches. In the last few years the study of pure cultures of bacteria, cultures and suspensions of protozoa, and short-term experiments with mixed rumen micro-organisms using radioisotopes have greatly increased our knowledge of the details of rumen metabolism. The results of some of these experiments will be described in the following sections. Again only a limited number of topics in which there have been recent advances have been selected for review.

Nitrogen metabolism

The fact that dietary proteins are broken down in the rumen and that much of the nitrogen from the protein is converted into microbial protein has been established by a number of workers. However, it was not until recently that the main bacteria responsible for proteolytic activity were identified. Blackburn & Hobson (1962) and Abou Akkada & Blackburn (1963) have shown that proteolytic activity is a property of certain strains of some species of rumen bacteria (for example, *Bacteroides amylophilus*, *Selenomonas ruminantium*, *Bacteroides ruminicola*, *Lachnospira multiparus*, *Butyrivibrio* spp. and Gram-positive cocci) which are also active in the degradation of plant carbohydrates. Some protozoa are also proteolytic (Abou Akkada & Howard, 1962; Williams, Davis, Doetsch & Gutierrez, 1961; Blackburn & Hobson, 1960*b*; Warner, 1956). That strains of a large number of rumen bacteria are proteolytic is in keeping with the finding that the proteolytic activity of rumen contents is not greatly dependent on composition of the diet, as most of the types of proteolytic bacteria are found in significant numbers in animals on a wide variety of diets. The majority of the proteolytic bacteria examined by Abou Akkada & Blackburn (1963) contained both exo- and endo-peptidases and produced a hydrolysate with a high content of amino acids and small peptides. The amino acid content of the rumen fluid is, however, low except during short periods after feeding, and the amino acids liberated during protein hydrolysis are rapidly removed (see, for instance, Annison, 1956). There is little evidence for absorption of amino acids through the rumen epithelium, but amino acids are utilized either directly or indirectly by the rumen organisms. The fact that no detectable concentration of a substance such as an amino acid or carbohydrate can be found in the rumen does not, of course, prove that it is not being utilized by the rumen bacteria. It may be utilized as fast as it is produced by some other microbial action. Although it is difficult to obtain quantitative data on the mode of utilization of amino acid nitrogen by the rumen organisms, recent studies have enabled some tentative conclusions to be drawn. From a consideration of experiments using the artificial rumen and mixed micro-organisms, Warner (1955) found that more cell protein appeared to be formed from the ammonia of the rumen fluid than from amino acids. Whilst peptides are growth stimulants for some bacteria (Bryant, Small, Bouma & Chu, 1958*b*; Huhtanen, 1955) and may be used for growth in preference to amino acids, recent work on pure cultures of bacteria has suggested that in fact many of the predominant rumen bacteria synthesize their

protein from ammonia, and that Warner's suggestion that ammonia is a major source of bacterial protein was correct. Ammonia is a normal constituent of rumen fluid and experiments with washed suspensions have shown that mixed rumen organisms will deaminate amino acids to give volatile fatty acids (including some branched-chain isomers) and ammonia. Annison (1956) showed that washed suspensions of rumen organisms incubated *in vitro* with casein formed (in molar proportions of fatty acids) 61% acetic; 19% propionic; 10% *n*-butyric; 4% *iso*butyric; 5% branched-chain valeric, and 1% *n*-valeric. The proportion of branched-chain acids normally found in rumen contents *in vivo* is much lower than this, probably owing to the rapid incorporation of these acids into cell material as described below. The degree of deaminative activity, unlike proteolytic activity, appears to depend to some extent on diet, but activity is found in all rumen contents. Recent pure culture studies (Bladen, Bryant & Doetsch, 1961; Abou Akkada & Blackburn, 1963) have shown that deaminative ability is possessed by certain strains of different species of rumen bacteria, but the activity is not as widely distributed as is proteolysis. Strains of *Bacteroides ruminicola*, *Butyrivibrio* and *Selenomonas ruminantium* and unclassified lipolytic bacteria are amongst the deaminating bacteria in the adult rumen. In the case of the selenomonads and lipolytic bacteria growing on glycerol as energy source, the concentration of ammonia produced from casein hydrolysate increased with growth of the bacteria, attaining a constant value at the time of maximum viable count of bacteria in the cultures. Ammonia production was not affected by the level of ammonia in the medium. Deamination is thus correlated with active growth of the bacteria (Hobson & Smith, unpublished) and this agrees with the increase in ammonia concentration found in the rumen after feeding when the increase in bacterial numbers is most rapid. Deamidation, an activity demonstrated in some strains of *Bacteroides ruminicola*, *Butyrivibrio*, *S. ruminantium*, *Eubacterium* and other rumen bacteria (Abou Akkada & Blackburn, 1963), as well as releasing ammonia from amides naturally occurring in feedstuffs, would also explain the ability of amides, such as propionamide, to replace part of the protein of feeds (Hale, 1956). Urease activity would also release ammonia in the rumen either from urea in the saliva or urea added to the feed. The rumen bacteria thus have ammonia, and, at least for a short time after the animal feeds, amino acids and peptides as sources of nitrogen. The further evidence in support of the hypothesis that ammonia nitrogen is used preferentially by many of the most important rumen bacteria has been provided by recent pure culture studies. The work of Bryant & Robinson (1961*b*, 1962*a*), Phillipson *et al.* (1962) and Abou Akkada & Blackburn (1963) has shown that many rumen bacteria, although able to utilize preformed amino acids when these are the sole source of nitrogen, when presented with a mixture of amino acids and ammonia appear preferentially to utilize the ammonia for synthesis of cell protein. Tests on pure cultures have also shown that some rumen bacteria have an absolute requirement for ammonia (see, for instance, Bryant & Robinson, 1961*b*). The main rumen bacteria thus seem to differ from some bacteria of other habitats in their preference for ammonia instead of preformed amino acids and this preference makes them especially suited to life in the rumen where there is a constant supply of ammonia. However, some strains of *Streptococcus bovis* which usually occur in the rumen in small numbers appear to fit into the normal pattern of preferring amino acids to

ammonia, although ammonia will serve as sole source of nitrogen (Wolin, Manning & Nelson, 1959).

There then remains the question of the source of carbon for cell synthesis. In the experiments of Abou Akkada & Blackburn mentioned above, a carbohydrate was included as energy source and the carbon of this could also serve as a source of cell carbon. There was also CO_2 in the gas phase. The experiments of Bryant & Robinson (1962*b*), in which a ^{14}C-labelled protein hydrolysate was used, showed that in the case of bacteria fixing large amounts of ammonia nitrogen the cell carbon must have come from carbohydrates or constituents of the medium other than amino acids. One medium constituent, which is also present in large amounts in the rumen, is carbon dioxide and another source of carbon in the rumen is the volatile fatty acids. Results published some years ago showed that ^{14}C from $^{14}CO_2$ could be incorporated into the protein of mixed rumen organisms (Otagaki, Black, Goss & Kleiber, 1955) or into some pure cultures of rumen bacteria (Huhtanen, Carleton & Roberts, 1954) and growth tests on many rumen bacteria show that they require CO_2 (see, for instance, Bryant & Burkey, 1953; Gill & King, 1958; Blackburn & Hobson, 1962). Branched- and straight-chain fatty acids have been shown to be growth factors for some rumen bacteria and also to stimulate cellulolysis by mixed rumen bacteria (see, for instance, Bryant & Robinson, 1962*a*; Hobson & Mann, 1961*b*; Wegner & Foster, 1960; Bentley, Johnson, Hershberger, Cline & Moxon, 1955; also Hobson & Smith, unpublished). More recent work has shown that the acids can be incorporated into the bacterial cells. In this work (Allison & Bryant, 1962; Allison, Bryant & Doetsch, 1962; Allison, Bryant, Katz & Keeney, 1962) a strain of *Ruminococcus flavifaciens* was used, but a similar incorporation of fatty acids probably occurs in other bacteria which are stimulated by these acids. The coccus was grown in the presence of ^{14}C-labelled *iso*valerate and ^{14}C was incorporated mainly into cell protein and lipid. The ^{14}C in the protein was all found in the leucine, showing that the function of the *iso*valerate was to serve as a carbon skeleton for leucine synthesis. Even when grown in the presence of protein hydrolysate or leucine the organism still synthesized most of its cellular leucine from *iso*valerate indicating that it had only a limited ability to incorporate exogenous amino acids. This was further borne out by the fact that the coccus incorporated only 2% of the ^{14}C when grown in uniformly labelled *Chlorella* protein hydrolysate compared with *Escherichia coli* which incorporated 37% of the ^{14}C. ^{14}C from *iso*butyrate ^{14}C was also incorporated into cellular valine and lipid (Wegner, personal communication to Allison, Bryant & Doetsch, 1962). Further studies showed that when grown in a medium containing casein hydrolysate, *iso*-butyrate, *iso*valerate and $^{14}CO_2$ (as carbonate), ^{14}C was incorporated into lipid (2% of cellular ^{14}C), nucleic acids (17%) and protein (77%). The label was found in fifteen amino acids of the hydrolysed cellular protein, and in the three branched-chain acids studied in detail the activity was all in the carboxyl carbon. It is thus suggested that CO_2 is a precursor of the carboxyl group of these amino acids. Similar studies by Wright (1960) on a strain of *Streptococcus bovis* isolated from the rumen showed that this organism incorporated CO_2 into the carboxyl group of aspartic acid even when growing in a medium containing casein hydrolysate with or without added aspartic acid. *S. bovis* is usually found in the rumen, but different strains vary in nutritional requirements, so that the behaviour of this strain of *S. bovis* may not

apply to all. The sulphur needed for the formation of the sulphur-containing amino acids can be supplied to the mixed rumen bacteria as sulphate, sulphide or elemental sulphur. Elemental sulphur was shown by Starks, Hale, Garrigus & Forbes (1953) to be utilized by lambs fed a semi-synthetic diet otherwise deficient in sulphur, as control lambs without added sulphur became ill and eventually died. Hale & Garrigus (1953) found that elemental ^{35}S fed to sheep appeared in the cystine, and possibly in the methionine of their wool. The work of Emery, Smith & Huffman (1957), Block, Stekol & Loosli (1951) and Anderson (1956) showed that sulphur from sulphate was incorporated into mixed rumen microbial protein as cystine and methionine, but the results of Müller & Krampitz (1955) suggested that only the bacteria incorporate sulphur. It is most likely, however, that sulphur from sulphate is not directly incorporated into the micro-organisms, but is first reduced to sulphide (Lewis, 1954; Anderson, 1956). Sulphide is also produced by the mixed rumen bacteria from sulphur amino acids in feed protein (Anderson, 1956), and such a production of sulphide has been noted in pure cultures of rumen bacteria such as *Selenomonas ruminantium*. Although sulphate is rapidly reduced in the rumen the main bacteria responsible for this have not yet been isolated, although small numbers of *Desulphovibrio* have been found (Coleman, 1960; Gutierrez, 1953). In pure culture studies Emery, Smith & Fai To (1957) found that only three out of ten strains of rumen bacteria studied incorporated appreciable sulphur from $^{35}SO_4$ and the one tested in detail, a strain of *Lachnospira multiparus*, preferentially used sulphur from cysteine to sulphur from sulphate. Work with *Bacteroides succinogenes* suggested that this cellulolytic bacterium is unable to utilize sulphate but will utilize sulphur from sulphide. The main form in which sulphur is normally utilized in the rumen is thus as sulphide and this can be provided either by reduction of sulphate or from sulphur amino acids of the feed. The sulphide sulphur is incorporated into bacterial protein as cystine and methionine and these amino acids, after digestion of the microbial protein in the intestines, can be absorbed and incorporated into body constituents such as wool (Hale & Garrigus, 1953) and milk (Block *et al.* 1951). There is, though, the possibility that sulphide sulphur is incorporated into body constituents by other pathways. Thus Anderson (1956) showed that sulphide was rapidly absorbed from the rumen into the blood stream and taken up by the liver but suggested that when only a small amount of sulphate is present in the feed it will be reduced to sulphide which will be incorporated into the micro-organisms to a much greater extent than it is absorbed into the blood stream.

Many rumen bacteria are thus able to incorporate a number of the simpler constituents of the rumen fluid into their cells and this ability has important consequences for the well-being of the host animal under adverse conditions. However, the bacteria still need a source of energy for growth and this is probably mainly provided by the carbohydrates of the feed. Lewis & McDonald (1958) showed that a carbohydrate was necessary for good utilization of feed protein. Cellulosic materials are present at all times in the rumen, even on intermittent feeding régimes, and hydrolysis of these would provide a slow release of simpler sugars which are probably available to other rumen bacteria. The slow hydrolysis of starch and the leaching of starchy polysaccharides and other oligo- and polysaccharides from plant material as the structure is disintegrated would also provide a further source of energy. However, the supply

of soluble carbohydrate must be limited and other sources of energy may be used by some bacteria. The end products of fermentation of sugars, such as lactate and possibly fatty acids, will also provide alternative sources of energy for some bacteria. Amino acids have been shown to act as sources of energy for some non-rumen bacteria (e.g. clostridia and some cocci), but, so far as the author is aware, only one rumen bacterium has been found to grow without a carbohydrate or non-amino organic acid as energy source (excluding methanogenic bacteria). *P. elsdenii* in washed suspension was shown by Lewis & Elsden (1955) to ferment L-serine, L-threonine and L-cysteine but growth on these substrates was not tested. However, Bladen *et al.* (1961) mentioned that a carbohydrate substrate was not needed for growth and ammonia production by *P. elsdenii* in a medium containing trypticase. In all other experiments on ammonia production from amino acids, utilization of ammonia, and proteolysis by growing strains of rumen bacteria a carbohydrate energy source has been needed. This correlates with the practice of feeding a carbohydrate along with urea or easily degraded protein to obtain maximum utilization of ammonia in the rumen. There may be bacteria in the rumen which can ferment amino acids, and which have not yet been isolated. Although rumen bacteria will grow in some media without added carbohydrate, growth is scanty and the presence of small amounts of carbohydrate or organic acids in the constituents of the media probably accounts for this growth. Purine fermentation could be another source of energy for some rumen bacteria as purines may comprise something like 45 % of the non-protein nitrogen of plants, and some have been shown to be degraded by mixed rumen bacteria *in vitro* (Jurtshuk, Doetsch & Shaw, 1958; Belasco, 1954). Purines have been reported to be growth factors for a strain of *Ruminococcus* (Ayers, 1958) but not to be energy sources. However, Whiteley & Douglas (1951) showed that one oral strain of *Micrococcus lactilyticus* (*Veillonella alcalescens*; *V. gazogenes*) could grow on hypoxanthine or xanthine as substrate and the same might apply to some rumen strains of this organism, as xanthine and usually hypoxanthine are amongst the purines of grass (Ferguson & Terry, 1953).

The ability of the rumen organisms to hydrolyse urea and use ammonia as a nitrogen source has been the basis of methods for substitution of the cheaper urea or ammonia for part of the protein of ruminant feedstuffs. Some 300 or 400 papers have been published on this topic and a list of relevant papers up to 1953 was given in *Feed Urea in Ruminant Nutrition*, a publication of the Allied Chemical and Dye Corporation, New York. A later review is that by Humphrey (1956). It seems that there is an optimum level of protein replacement. A source of carbohydrate is also needed for best utilization of the urea or ammonia, and molasses is one additive that has been extensively used. Urea has also been used to supplement the poor pasture available to animals in some parts of the world during dry seasons. The urea, usually with molasses added, has been sprayed on the pasture so that it is taken by the grazing animals, or it has been added to cut grass or hay and stall-fed. Since large quantities of urea are toxic to ruminants when rapidly ingested, most likely due to excess formation of ammonia, care has to be taken to see that the urea dressing is not too heavy and that if the animals have been on a poor feed for some time that they are gradually accustomed to the urea intake. Another way of ensuring that the urea is not taken too rapidly is to incorporate urea and molasses in a salt lick (Louw,

1960). Some recent papers on supplementation of poor quality pasture with urea are those by Beames (1959), Fels (1959), Messenger (1960), Willoughby & Axelsen (1960). Recent papers on urea toxicity are those by Coombe *et al.* (1960), and Lewis (1960). Since rumen bacteria can synthesize vitamins of the B complex, including B_{12}, and microbial protein is of good nutritional value the microbial protein passing from the rumen for digestion in the alimentary tract makes good any deficiencies in the dietary supply of B vitamins or amino acids.

Urea is a normal constituent of the saliva and although the amount of nitrogen entering the rumen from this source is of little significance compared with the dietary nitrogen on rations of high protein content, under some circumstances where the diet is low in protein, such as on the poor pasture mentioned above, the salivary urea can provide a significant amount of nitrogen for the bacteria to enable them to make increased use of the carbohydrate available in the forage. Secretion of urea into the rumen via the epithelium could provide additional nitrogen for the bacteria. The amounts and nature of secretions into the rumen are dealt with in more detail in Part I.

Bacterial lipids

In the previous section the incorporation of volatile fatty acids into cell protein was discussed and it was mentioned that these acids were also incorporated into cell lipid. The lipids of the bacteria may be set free during digestion of the cells in the intestines and the lipids could be absorbed and incorporated into body lipids of the host. It has been known for some time that unsaturated fatty acids of dietary lipids are hydrogenated by the rumen micro-organisms, and to this observation has been ascribed the fact that the depot fats in cows and sheep are more saturated than those of other herbivores, for instance, horses, consuming the same pasture lipids. The hydrolysis of feed lipids by rumen organisms has also been established (Garton, Hobson & Lough, 1958; Dawson, 1959; Garton, Lough & Vioque, 1961; Wright, 1961; Zerebcov & Solncev, 1962) and one strain of anaerobic rumen bacteria capable of hydrolysing linseed oil has been obtained (Hobson & Mann, 1961*b*). Acetate formed by rumen bacterial fermentations is also a precursor of milk fats. The subject of lipid metabolism in ruminants and herbivores in general has been reviewed recently by Shorland & Hansen (1957) and by Garton (1960, 1961) and a further review on lipid metabolism will be presented shortly in *J. Lipid Research.* However, a short discussion of the recent papers on synthesis of lipids by rumen bacteria is relevant here.

Hansen, Shorland & Cooke (1955, 1956) and Shorland, Gerson & Hansen (1955) found small quantities of odd numbered carbon and branched-chain fatty acids in ruminant milk and body fats. The results of recent work (which is well reviewed and documented in the paper by Keeney, Katz & Allison (1962)) have thrown some doubt on the idea that these acids originate in the ruminant tissues.

Keeney *et al.* (1962) showed that there is a high level of odd-numbered carbon and branched-chain fatty acids in the lipids of mixed bovine rumen bacteria, and that these acids could be detected in blood serum lipids. They are thus available for incorporation into milk fat by the mammary gland. These authors also calculated that even on the basis of their experiments, which did not extract the total amount of microbial lipid in the rumen contents, about 142 g of protozoal and bacterial lipid

would pass to the intestines each day, and, as the cow was producing about 500 g of butterfat each day, it was suggested that a significant amount of milk lipids could come from microbial lipids. They also calculated that the amount of C_{15} branched-chain fatty acids in the microbial lipid passing to the intestine could account for more than half the C_{15} branched-chain acid in the butterfat secreted. The origin of the bacterial lipid is shown by the work of Allison, Bryant, Katz & Keeney (1962). On p. 300 the incorporation of branched-chain volatile fatty acids into bacterial protein was discussed and in these experiments some of the radioactive carbon from ^{14}C isovalerate was found in the cell lipids. When grown in the presence of 1-^{14}C-isovalerate one strain of *Ruminococcus flavifaciens* incorporated most of the label found in the lipid into a branched-chain C_{15} fatty acid with some in a C_{17} acid and also an appreciable amount in a C_{15} branched-chain aldehyde. A strain of *R. albus* did not incorporate ^{14}C from labelled isovalerate but when grown in 1-^{14}C-isobutyrate incorporated most of the ^{14}C into the cell lipid mainly as C_{14} and C_{16} branched-chain acids, with some as C_{14} and C_{16} aldehydes. Wegner & Foster (1961) also found that a strain of *Bacteroides succinogenes* incorporated ^{14}C from 1-^{14}C-isobutyrate or 1-^{14}C-valerate into lipid, and almost all the label was found in fatty acid carbon of the phospholipid phosphotidylethanolamine. The structure of the fatty acid was not reported. A rumen *Borrelia* sp. also incorporated *iso*butyrate and valerate into cell lipid. Similar results may be found with other rumen bacteria which need volatile fatty acids as growth factors and there seems no doubt that rumen bacteria, digested in the intestines, are the source of a considerable amount of lipid material for the host animal. Parks, Keeney & Schwartz (1961) found that branched-chain aldehydes were amongst the major bound aldehydes in butter oil and it seems possible that the source of these aldehydes is the rumen bacterial lipids. Whether the protozoa are able to synthesize all of their lipids, or whether some of them come from ingested bacteria, is not established, but in either case the net results to the host animal would be the same.

Thus apart from fermentation products and vitamins the rumen bacteria contain protein and lipid materials which can be incorporated into the tissues of the host after digestion of the microbial cells in the abomasum and intestines.

The metabolism of some primary fermentation products

The rumen is a complex mixed culture of micro-organisms pursuing a range of fermentations, some organisms of primary importance in the breakdown of feed materials, others causing secondary fermentations continuing the digestive processes to give products of value to the host, but also helping to maintain the stability of the rumen culture and possibly to increase the rates of primary hydrolysis and fermentation by removing some of the end products. There is no doubt that some bacteria and protozoa will live on the end products of the polysaccharide hydrolysis by other organisms and the microscopic observation of two organisms in contact with an insoluble substrate does not mean that both will attack the substrate. For instance, *in vitro* it was found that *Borrelia* tended to migrate through an agar medium to grow on the products of cellulose hydrolysis by *Bacteroides succinogenes* (Bryant, 1952). Apart from this use of hydrolysis products of polysaccharides there is a turnover of the primary fermentation products of carbohydrates. The main acids normally

present in the rumen are acetic, propionic and butyric. However, in addition to these, lactic, succinic and formic acids, hydrogen, carbon dioxide and ethanol are all products of fermentation of carbohydrates by rumen bacteria and protozoa *in vitro*. Although it is difficult to make firm statements about the products of fermentation *in vivo* from batch culture *in vitro*, because the amounts and nature of fermentation products can be influenced by such things as the pH and composition of medium (see, for instance, Lee & Moore (1959) for butyrivibrio), all these products seem to occur at least transitorily in the rumen. Carbon dioxide, we have already seen, can be incorporated into bacterial cell material, but ^{14}C-labelled CO_2 added to rumen contents *in vitro* also appears in volatile fatty acids (Van Campden & Matrone, 1960; Otagaki *et al.* 1955). Carbon dioxide can also be combined with hydrogen to form methane. A bacterium which uses this reaction, *Methanobacterium ruminantium*, has been isolated from the rumen (Smith & Hungate, 1958). Viable counts of this bacterium in the rumen, combined with *in vitro* measurement of the rate of combination of carbon dioxide and hydrogen, have shown that it is present in the rumen in numbers more than sufficient to account for the rate of production of methane *in vivo*. Rumen contents as a whole have a large capacity for utilizing hydrogen and it thus seems that the hydrogen produced by fermentation reactions would be almost instantaneously used in the production of methane or reduction of sulphate. A further carbohydrate fermentation product which is a precursor of methane is formate (see, for instance, Beijer, 1952; Matsumoto, 1961). Rumen contents have a capacity to utilize formate at a rate greater than that at which it might be expected to be produced by fermentations (Carroll & Hungate, 1955). *Methanobacterium ruminantium* besides using CO_2 and H_2 will also use formate as a substrate for production of methane. Formic acid produced by fermentation of carbohydrate would thus be expected to have a very short existence in the rumen.

Lactic and succinic acids usually have only a transitory existence in the rumen and are readily converted to volatile fatty acids. It has often been assumed that propionic acid is the major product of lactic acid fermentation in the rumen, but acetic acid is also formed, and the work of Jayasuriya & Hungate (1959) and Bruno & Moore (1962) suggests that acetic acid is usually the main product. The ratio of acetic to propionic acid does, however, vary somewhat with diet and the results of Baldwin, Wood & Emery (1962) suggested that the ratio of acetate to propionate decreased with increasing dietary carbohydrate.

Recent studies on the pathway of breakdown of lactic acid by mixed rumen bacteria have posed the question as to the organisms mainly responsible for lactate metabolism in the rumen. There are two pathways of lactate conversion to propionate now known in bacteria. One is via succinate, the other via acrylate. The experiments of Baldwin *et al.* (1962) have suggested that the main pathway of lactate metabolism by mixed rumen bacteria is the acrylate one. A number of rumen bacteria are known to ferment lactate in pure culture, but of the bacteria examined in detail only *Peptostreptococcus elsdenii* ferments lactate via the acrylate pathway (Ladd & Walker, 1959). However, this bacterium does not occur in large numbers in the normal adult rumen. A bacterium which is generally present in the rumen in high numbers is *Selenomonas ruminantium* (Bryant, 1956; Hobson & Mann, 1961*b*). One variety of this ferments lactate, but it does so via the succinate pathway

(Paynter, 1962), as do the propionibacteria and *Veillonella gazogenes* which are present in the rumen in small numbers (Johns, 1951*a*, *b*). It may be that some of the other lactate-fermenting bacteria, not so far studied in detail, may ferment lactate via acrylate, and be the main lactate-fermenters in the rumen. However, the results of Jayasuraya & Hungate (1959) suggest that lactate is not an important intermediate in propionate production in the rumen of animals on normal feeds and the small numbers of lactate-fermenters such as *Peptostreptococcus elsdenii* may be sufficient to account for all the propionate formed by this reaction. Of course, on some diets containing large amounts of starchy concentrates such as flaked maize, or on silage feeds, where large amounts of lactic acid might be expected, the observations made above might not hold. However, it would appear that succinate is usually of much more importance than lactate as a precursor of ruminal propionic acid. Succinic acid is produced by many rumen bacteria and succinate decarboxylation is carried out by washed suspensions of mixed rumen bacteria (Johns, 1951*c*; Sijpesteijn & Elsden, 1952) at an optimum pH of 6·2, with considerable activity between pH 5 and 7. This optimum is near the usual pH of rumen contents. The main bacteria responsible for succinate metabolism are not known at the moment, although probably *Veillonella gazogenes* plays some part as it is generally present in the rumen, although not in large numbers (about 10^6/ml), and it rapidly decarboxylates succinate at an optimum pH of 6. It seems unlikely that propionibacteria play much part in this reaction as they are usually present only in small numbers (the results of Gutierrez (1953) appear to be an exception), and the optimum pH of succinate decarboxylation by organisms of this genus is 5–5·2 and decarboxylation is very slow.

Ethanol is produced *in vitro* by some pure cultures of rumen bacteria (e.g. strains of *Ruminococcus*), although it is not usually found in the rumen, and ethanol has been used along with urea as a feed supplement for cattle (Morris & Horton, 1959). If ethanol is fermented by the rumen bacteria its products have not been identified. One possibility is that it is used along with acetic acid to form butyric acid, and one bacterium capable of bringing about this reaction has been isolated from the rumen (Gray, 1958). However, Lewis, Emery & Everett (1958) found that ethanol was not fermented *in vitro* by rumen contents from steers, but that it disappeared from the rumen *in vivo*, presumably by absorption as the ethanol content of the blood increased 2–4 h after ethanol had been added to the rumen. Small amounts of ethanol are probably fermented rather than absorbed and, although the bacterium which fermented ethanol and acetic acid, *Clostridium kluyverii* (Gray, 1958), was found only in small numbers, as the amount of ethanol formed from fermentation of carbohydrates is probably small a large number of bacteria capable of utilizing it would not be expected. The bacterial populations in animals fed urea and ethanol are probably quite different from those of the animals used in the experiments mentioned above and a greater utilization of ethanol presumably takes place.

Methanol is formed during degradation of pectic substances and is slowly metabolized by sheep rumen contents *in vitro*, but the products of its decomposition have not been identified (Howard, 1961).

Only one organism (*Peptostreptococcus elsdenii*) is definitely known to produce fatty acids higher than butyric (valeric and caproic) by direct fermentation of carbohydrate. Although traces of higher fatty acids have sometimes been found in our

own laboratories in *in vitro* cultures of some rumen bacteria fermenting carbohydrate, the possibility that they come from degradation of amino acids in the media has not been excluded. It seems probable that the main sources of higher fatty acids *in vivo* are amino acids. However, the possibility of synthesis from lower fatty acids does exist and Gray, Pilgrim, Rodda & Weller (1952) and Van Campden & Matrone (1960) showed that acetate could be converted to butyrate, and propionate to valerate by mixed rumen micro-organisms. Amongst the pure cultures of rumen bacteria studied butyrivibrio strains will utilize acetic acid, presumably in the formation of butyric acid. Other rumen bacteria are stimulated by small amounts of lower fatty acids, although some of the acids may be incorporated into cell constituents and not be the precursors of higher acids (see pp. 299, 300.)

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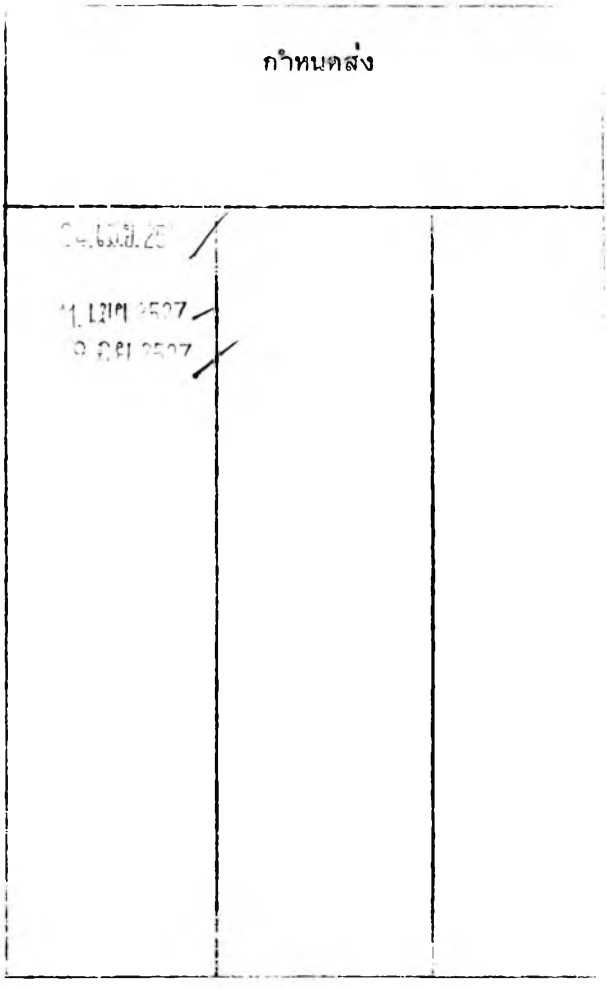
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Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)